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RELATION BETWEEN THE ELECTRONIC STRUCTURE AND CARCINOGENIC ACTIVITY OF 4-NITROQUINOLINE N-OXIDE AND RELATED COMPOUNDS

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INTRODUCTION

Although about thirty years have elapsed since the discovery of the pure chemical carcinogens such as polycondensed aromatic hydrocarbons and azo compounds, little is known about the nature of the carcinogenic process caused by these compounds. Of course this may principally be attributed to the difficulty of clarifying the successive changes in the genesis of tumors in the body. But the complexity of chemical structure and reaction of these carcinogens may also be responsible for these circumstances. In this connection the recent finding by Nakahara, Fukuoka and Sugimura (1) that 4-nitroquinoline N-oxide possesses a potent skin cancer producing activity provided a powerful tool for the study of carcinogenesis because its chemical structure and reaction were considerably simpler than those of the chemical carcinogens hitherto reported.

The most probable chemical reaction of 4-nitroquinoline N-oxide with a nucleophilic reagent might be the replacement of nitro group at the position 4 owing to the extreme electron-attracting nature of N-oxide group. Hence the biological action of this compound might have a correlation to this reaction. Okabayashi (2), who studied experimentally the antifungal activity of this compound on *Aspergillus*, demonstrated that the nitro group of this compound could easily be displaced by nucleophilic group such as SH under physiological conditions, and assumed that its antifungal action might be intimately connected with this substitution reaction.

Nakahara *et al.* (3, 4), from the investigation of the structure-carcinogenic activity relationship, suggested that the carcinogenic action of 4-nitroquinoline N-oxide may be due to the nucleophilic substitution at position 4. In connection with this proposition, the following relation found by Endo (5) through his *in vitro* experiment seems to be very interesting. That is, with respect to 4-nitroquinoline N-oxide and related compounds, the carcinogenic activity had a distinct relation with the easiness of nucleophilic substitution of nitro by sulfhydryl of cysteine or glutathione.

The present authors have studied the correlation between the electronic structure and carcinogenic activity of polycondensed aromatic hydrocarbons and azo compounds

(10, 11, 15, 20). In the light of these theoretical studies they have proposed a conclusion that the reaction of primary importance in carcinogenesis due to chemical substances of all types might be the attack of active region of carcinogens on a nucleophilic center in the body (11, 15, 16, 17, 18, 19, 20).

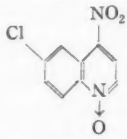
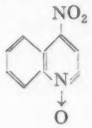
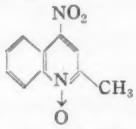
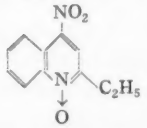
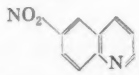
In view of the results stated above, it might be worth while to investigate the electronic structure of nitroquinoline compounds. Some of the present authors have presented the frontier electron theory as one of the methods for predicting the chemical reactivity of conjugated molecules (6, 7, 8, 9). The theory has also been proved to be successful in explaining the carcinogenic activity of non-substituted aromatic hydrocarbons (10), heterocyclic aromatic compounds (11), methyl substituted 1,2-benzanthracene and aromatic azo compounds (11) as well as the plant growth activity of benzoic acid derivatives (12) and naphtoic acid derivatives (13). In the present paper the electronic structure of 4-nitroquinoline N-oxide and related compounds were investigated by the frontier electron method and the result was discussed in comparison with the experimental reactivity towards SH group and also with the carcinogenic activity.

Theoretical Indexes and Parameters Used in the Calculation

Theoretical indexes used in this report are the frontier electron density and the approximate superdelocalizability. They have both been established as good indexes of easiness not only for substitution and addition but also for molecular complex formation. The details of these indexes were given in our previous papers (6, 7, 8, 9, 11).

The values of the Coulomb integral of nitrogen of 4-nitroquinoline and 6-nitroquinoline, and the Coulomb and the resonance integrals for methyl, nitro, and chloro groups were taken as equal to those used in our previous papers (11, 12). The Coulomb integrals of nitrogen and oxygen atoms of N-oxide group, denoted respectively by $\alpha+\delta_1\beta$ and $\alpha+\delta_2\beta$, where α and β are the Coulomb and the resonance integrals in benzene, could not unquestionably be determined. However, the electronegativity of the nitrogen atom in this group is far larger than that of the pyridine-type nitrogen. Hence it may be reasonable to adopt a relatively large positive value of δ_1 for the nitrogen atom. On the other hand, the value of δ_2, of the oxygen atom of N-oxide group, might be smaller than that of furan-type oxygen because of the small electronegativity of the former. From these considerations, we adopted tentatively the values of δ_1 and δ_2 as 3.5 and 0.6, respectively. Sliding of these values to some extent does not cause a serious change of the result. The Coulomb integral for ethyl group and the carbon atom adjacent to this were taken as $\alpha+2.9\beta$ and $\alpha-0.1\beta$, respectively. The latter value was the same as that of methyl group. The former was adopted, in comparison with the value $\alpha+3.0\beta$ for methyl group (11,

Table 1. Comparison of the theoretical reactivity indexes for nucleophilic reaction with carcinogenic activity of 4-nitroquinoline N-oxide and its related compounds.

Compounds	Approximate superdelocalizability	Frontier electron density	Amperometric titration*		Skin cancer producing activity**
			Cysteine	Glutathione	
 6-chloro-4-nitroquioline N-oxide	3.361	0.189	0.012	0.017	+
 4-nitroquinoline N-oxide	3.113	0.186	0.050	0.030	+
 4-nitroquinaldine N-oxide	1.913	0.178	0.150	0.132	+
 2-ethyl-4-nitroquinoline N-oxide	1.894	0.177	0.173	0.150	+
4-nitroquinoline	1.579	0.176	0.180	0.160	−
4-nitropyridine N-oxide	1.092	0.149	0.207	0.170	−
 6-nitroquinoline	0.173	0.046	0.223	0.175	−

* See reference 5. ** See references 1, 3 and 4.

12), referring to the I'haya's calculation (14) of the electron distribution of toluene and ethyl benzene.

Result and Discussion

In Table 1, the values of frontier electron density and approximate superdelocalizability for nucleophilic reaction at the position 4 (position 6 for 6-nitroquinoline) and the easiness of nucleophilic replacement of nitro group with sulfhydryl of cysteine and glutathione were compared. The figures in the fourth and the fifth columns are the result of amperometric titration, indicating the amount of $AgNO_3$ corresponding to the remaining SH group after the reaction of these nitroquinoline compounds with cysteine or glutathione under physiological condition for one hour. Therefore the smaller the value of a compound is, the more reactive the compound is. From Table 1, we can see a strikingly good parallelism between the experiment and the theoretical indexes.

Furthermore, the relation between the carcinogenic activity and the theoretical indexes is seen in Table 1. In the sixth column, the skin cancer producing activity was listed. The signs (+) and (−) represent carcinogenicity and non-carcinogenicity, respectively. Comparing the carcinogenic activity and the theoretical indexes, one would instantly notice a definite relation between them. Although it was impossible to correlate them quantitatively because of the qualitative nature of magnitude of carcinogenic activity, the values of theoretical indexes larger than a certain threshold seem to be needed for occurrence of carcinogenic activity.

After the study of the correlation between the electronic structure and carcinogenic activity of polycondensed aromatic hydrocarbons and azo compounds we have concluded that the interaction between the reactive region of these carcinogens with nucleophilic center in the body may primarily be concerned with the switch turning the normal cell to malignant growth (11, 15, 16, 17, 18, 19). The results obtained in the present report would provide an additional support to this supposition.

As the nucleophilic center, SH group was cited exclusively in this report because there was no other experimental data available to correlate with theory. This does not mean, however, that the nucleophilic center which might be concerned with the production of tumors is SH group itself. Many other nucleophilic groups exist in the body and at the present stage of knowledge it is difficult to decide which of them is actually connected with the carcinogenesis.

Summary

The electronic structure of 4-nitroquinoline N-oxide and related compounds were investigated. The frontier electron density and the approximate superdelocalizability for nucleophilic reaction at the carbon atom to which nitro group is attached had a

definite correlation with the carcinogenic activity. This is a support for our proposition that the interaction with the nucleophilic center in the body might be primarily concerned with the production of cancer by all types of chemical carcinogens.

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SUMMATION OF CARCINOGENIC EFFECTS OF CHEMICALLY UNRELATED CARCINOGENS, 4-NITROQUINOLINE N-OXIDE AND 20-METHYLCHOLANTHRENE*

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INTRODUCTION

Whether or not the carcinogenic process started by one carcinogen can be brought to completion by another carcinogen of different chemical nature is a question of some importance, for upon it may rest the fundamental principle that the irreversible alterations in the cell induced by different agents to produce cell cancerization may be qualitatively alike. Previous experiments by Hieger (1) and Lavik et al. (2), using the combination of 1,2-benzpyrene and 1,2,5,6-dibenzanthracene and showing them to be mutually substitutable in their carcinogenic action, were of little value, since the structure and hence presumably chemical reactions of the two carcinogens used were so similar that it might have amounted to simply giving larger doses of a single carcinogen. Other combinations tried by other investigators, because of the difference in the target tissues of the carcinogens involved or of other reasons, failed to give a conclusive evidence. The recent discovery of a new potent carcinogen, 4-nitroquinoline N-oxide (Nakahara, Fukuoka and Sugimura (3)), with the same target tissue as, but of strikingly different chemical structure from the carcinogenic hydrocarbons, rendered it possible to approach the subject on a more competent basis.

We now know that the carcinogenic action of 4-nitroquinoline N-oxide is probably dependent on the reactivity of the nitro-group at position 4 (4) and that as soon as this nitro-group underwent substitution reaction with SH-compounds the carcinogenicity of the molecule is not only lost but the products of the reaction are all non-carcinogenic. This last finding which showed that the reaction itself, not any chemical product of the reaction, may be the proximate cause of carcinogenesis, led us to suggest that the energy released in the reaction may produce misconstruction of cell duplicants (nucleic acid) by interfering with their replication process (5). This situation is in marked contrast to the case of hydrocarbons, where the

* Preliminary report on this study was published by Nakahara, W., and Fukuoka, F.: Summation cancerogener Wirkungen von chemisch verschiedenartigen Cancerogenen. Naturwissenshaften, 47: 44-45 (1960).

complex chemical structure is preventing investigators from arriving at a satisfactory agreement even as to the carcinogenically active region of the molecule, to say nothing of the precise nature of the chemical reactions involved.

The experiments to be reported in this paper were originally planned with the hope of determining whether or not the identical immediate mechanism can be assumed for carcinogens of different chemical groups. We reasoned that if two chemically unrelated carcinogens were applied one after the other and found capable of acting as though they were one, it would prove the identity of their proximate carcinogenic mechanism: If they accomplished carcinogenesis through different mechanisms, their respective actions being qualitatively different, could not be capable of summation to each other. For the purpose of the experiments we chose 20-methylcholanthrene as the partner carcinogen to be used in combination with 4-nitroquinoline N-oxide. The results showed that the carcinogenic process started by either of the two substances can indeed be completed by the other, affording clear evidence that their effects are capable of summation, and consequently that perhaps their immediate carcinogenic mechanism may be qualitatively identical.

Experimental Methods

The plan of the main experiments was to observe the tumor yields after the two carcinogens were applied to the skin of mice, a period of treatment with one carcinogen followed by that with the other, holding the doses of both carcinogens at the level where either of them alone does not produce tumors. The doses of carcinogen which can be delivered without actually producing tumors in the treated animals may be spoken of as *submanifestational doses*, rather than as subcarcinogenic doses, since, as will be revealed later, such doses do produce carcinogenic effects which, however, are inadequate to manifest themselves in the form of actual tumor production.

Both 4-nitroquinoline N-oxide and 20-methylcholanthrene were used as 0.25 percent solution in benzene, and as accurately as possible of 1/50 cc of the solution, equivalent of 0.05 mg of the carcinogen, was delivered by means of a small pipette and allowed to spread over the same skin area at each application. The area of the skin at the interscapular region was depilated beforehand for the treatment, which was given at the rate of three applications a week. Mice used were adult males of dd strain, and all the experimental procedures were carried out as nearly the same as practicable for all the groups.

The diagnoses of all the tumors recorded were confirmed by histological examination.

Submanifestational Doses of 4-Nitroquinoline N-oxide and 20-Methylcholanthrene

The first series of experiments was dovoted to determining the submanifestational doses of 4-nitroquinoline N-oxide and 20-methylcholanthrene.

4-Nitroquinoline N-oxide.

Through past experience we are aware that 4-nitroquinoline N-oxide, when applied to the skin of mice at the rate of three times a week in 0.25 percent solution in benzene, will begin to produce progressive papillomas in 70 to 100 days, which may be taken that 30 or more applications will result in the manifestation of tumor formation. We, therefore, tested the effect of the substance at two lower dosage levels, namely, 10 and 20 applications.

Experiment 1. (Chart 1, A). Mice were given 10 applications of 4-nitroquinoline N-oxide at the rate of 3 times a week, and were kept under observation for 200 days thereafter with no further treatment. One mouse showed a small papilloma, which spontaneously regressed, and the treated area of the skin remained normal to all appearance. No tumor was found in any of the 14 mice that survived long

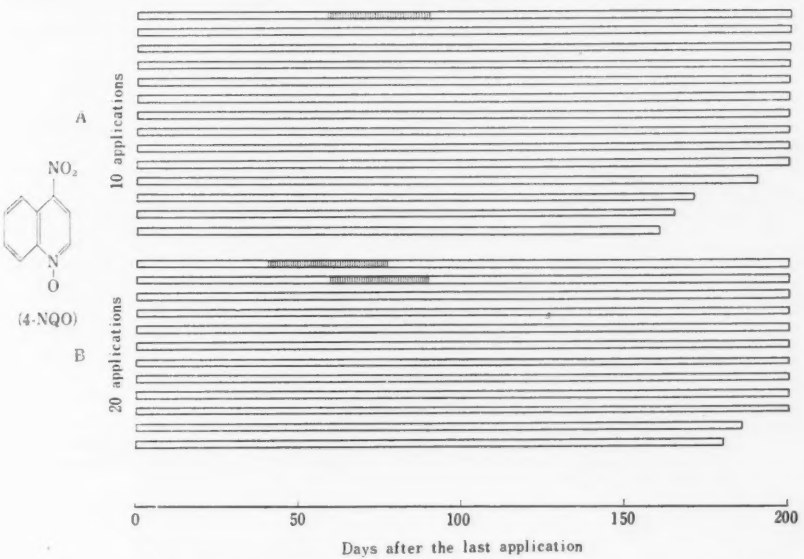

Chart 1. Submanifestational effect of 4-nitroquinoline N-oxide. A. Result of 10 applications. B. Result of 20 applications. In this and three other charts to follow (Charts 1-4) each horizontal bar represents a mouse, the shaded part showing the period in which papilloma was present. 10 or 20 applications may be regarded as submanifestational dose for 4-nitroquinoline N-oxide.

enough to be experimentally effective.

Experiment 2. (Chart 1, B). Effect of 20 applications of 4-nitroquinoline N-oxide was tested in this experiment, with the same negative result as in Experiment 1. Twelve mice survived 200 days or so after the last application of the substance. Earlier in the course of the experiment two mice showed very small papillomas, but these completely regressed after a relatively short time and at the end of experimental period the skin area appeared entirely normal.

The above experiments established that 10–20 applications may be safely regarded as submanifestational doses for 4-nitroquinoline N-oxide.

20-Methylcholanthrene.

In a similar manner 20-methylcholanthrene was tested at two dosage levels.

Experiment 3. (Chart 2, A). Mice were given 10 applications each of 0.25 percent solution in benzene of 20-methylcholanthrene at the rate of 3 applications a week and were kept under observation for 200 days after the last application. Small papillomas were seen in a few of the mice during 50–80 day period, but they all spontaneously regressed and completely disappeared, except in a single mouse, in

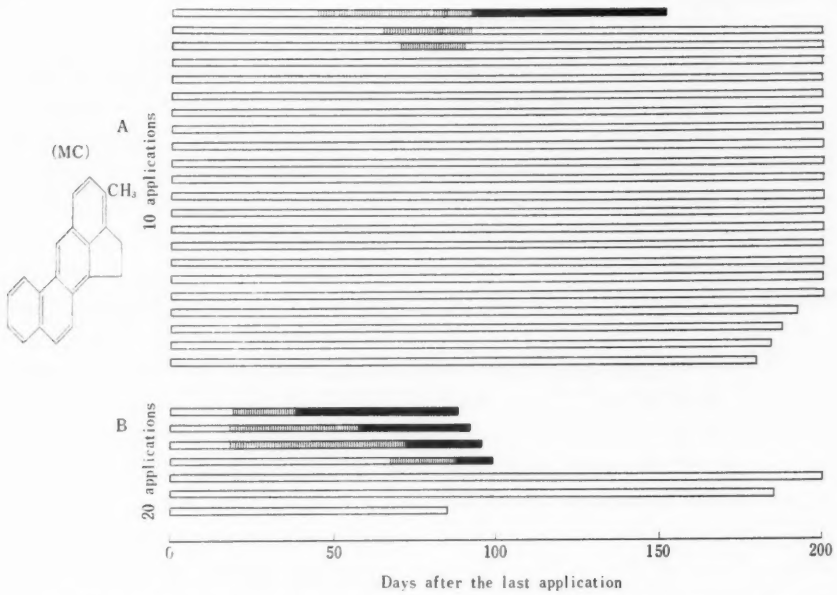

Chart 2. Effect of 10 (A) and 20 applications (B) of 20-methylcholanthrene. The black part of the bar shows the period in which was present a tumor clinically diagnosed as malignant. The diagnosis was confirmed by histological examination at autopsy. 10 applications may be considered as submanifestational dose for 20-methylcholanthrene.

which the growth progressed gradually and established itself as a malignant tumor in about 100 days. The final result showed positive tumor production in 1 out of 22 mice, indicating that for any practical purpose 10 applications may be regarded as the maximum submanifestational dose for 20-methylcholanthrene.

Experiment 4. (Chart 2, B). In this experiment the effect of 20 applications of 20-methylcholanthrene was tested, also at the rate of 3 applications a week. Four out of 7 mice in this group showed definite papillomas in 20-27 days, and these all grew progressively into malignant tumors.

The above results clearly demonstrated that on the percent concentration basis 20-methylcholanthrene is a stronger carcinogen than 4-nitroquinoline N-oxide. This fact has an important theoretical implication as will develop later in our discussion.

Summation of Effect of 4-Nitroquinoline N-oxide and of 20-Methylcholanthrene

We now come to the critical experiments in which a period of treatment with one carcinogen was followed by that with the other, both carcinogens being applied in submanifestational doses.

Experiment 5. (Chart 3, A). 10 mice were treated with 10 applications of 20-

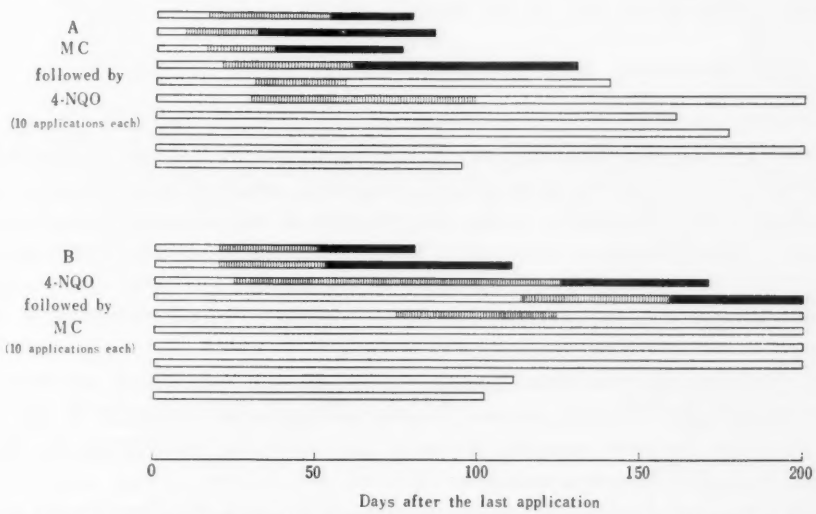

Chart 3. Effect of submanifestational dose of one carcinogen followed immediately after submanifestational dose of the other carcinogen. A. 20-methylcholanthrene followed by 4-nitroquinoline N-oxide. B. 4-Nitroquinoline N-oxide followed by 20-methylcholanthrene. The shaded and black parts of the bar show the presence of papilloma and carcinoma respectively.

methylcholanthrene and then with 10 applications of 4-nitroquinoline N-oxide, all at the rate of 3 applications a week in a continuous series.

As shown in Chart 3, A, as many as 6 of the 10 mice developed papillomas during 20 to 40 days after the last application of 4-nitroquinoline N-oxide. In 2 of these cases papillomas later regressed, but in 4 they continued to grow and produced typical skin carcinomas. The final tumor yield was therefore 4 out of 10.

Experiment 6. (Chart 3, B). In this experiment, simply the order of applications of the two carcinogens was reversed, mice being treated first with 10 applications of 4-nitroquinoline N-oxide and then with 10 applications of 20-methylcholanthrene. Ten mice were used. The rate of the development of papillomas and eventual carcinomas in this experiment is represented in Chart 3, B, from which it may be seen that the growths appeared generally later than in Experiment 5, although the final yield of malignant tumors was the same (4 out of 10 mice).

It may be noted that when the treatment with 20-methylcholanthrene preceded that with 4-nitroquinoline N-oxide, the rate of tumor production was perceptibly more rapid than when the order of the treatments was reversed, and yet the final tumor yield was not materially different. These results are consistent with the fact that in the dosage used 20-methylcholanthrene is more powerful than 4-nitroquinoline N-oxide, and at the same time they support the conclusion that the final result of the summation of each effect is the same as may be expected.

Irreversibility of Submanifestational Carcinogenic Effect

Although the phenomenon of carcinogenesis as a whole is admittedly due to an irreversible cell alteration, it does not necessarily follow that each of the steps leading to the final result must also be irreversible, and the case must be decided by direct experiment evidence. The demonstration of the submanifestational effect of two chemically different carcinogens in the preceding experiments afforded an opportunity for testing the irreversibility of the induced cell state, which is submanifestational and yet is capable of being brought to tumor production by the summation with the effect of another carcinogen.

In the following experiments an interval of 200 days was allowed between the first and second sets of treatment in order to determine the effect, if any, of the prolonged period of non-treatment on the persistence of the submanifestational effect produced by the first treatment.

Experiment 7. (Chart 4, A). In this experiment mice were first given 20 applications of 4-nitroquinoline N-oxide at the rate of 3 applications a week (submanifestational dose). No tumor developed in any of the mice, as may be expected. 200 days after the last application, the mice were challanged with a submanifestational dose of 20-methylcholanthrene, namely, 10 applications at the rate of 3 times a week.

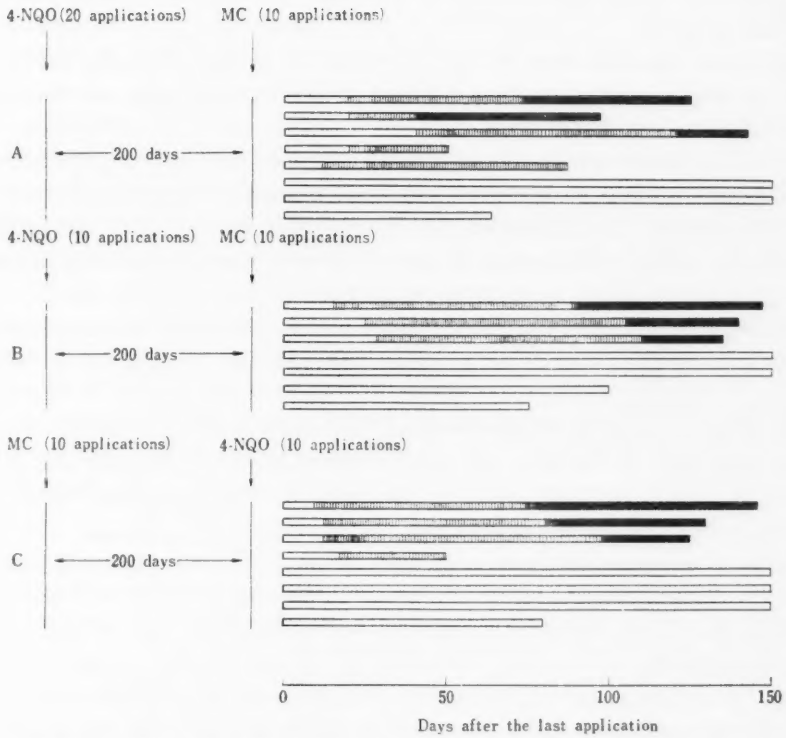

Chart 4. Effect of submanifestational dose of one carcinogen followed by submanifestational dose of one carcinogen followed by submanifestational dose of another carcinogen, *with the interval of 200 days between the two doses.* A and B, 4-nitroquinoline N-oxide followed by 20-methylcholanthrene. C. 20-methylcholanthrene followed by 4-nitroquinoline N-oxide.

In 8 mice, for which the test has been completed, as many as 5 responded with papilloma production during 20 to 40 days after the last application of 20-methylcholanthrene, as shown in Chart 4,A. Two of these mice died with merely papillomas, too early to tell whether the papilloma would have progressed into malignant state. In the remaining three, tumors became definitely malignant before the mice died.

The result seems unequivocal in showing that the effect produced by a submanifestational dose of 4-nitroquinoline N-oxide persists and remains capable of summation for at least 200 days.

Experiment 8. (Chart 4, B). A similar experiment to the above was repeated, using, however, 10 applications, instead of 20, of 4-nitroquinoline N-oxide in the first place. A rest period of 200 days was allowed as in Experiment 7, between the

first treatment with 4-nitroquinoline N-oxide and the second treatment with 20-methylcholanthrene.

Seven mice remained experimentally effective: 3 of them developed papillomas within 30 days after the last application of 20-methylcholanthrene, and these progressed to carcinoma in due time.

The slightly lower yield of tumors in this experiment than in the one immediately preceding may possibly be due to the fact that the primary submanifestational dose of 4-nitroquinoline N-oxide used was 10 applications in this and 20 in the preceding experiments, although the number of mice is too small and the difference in tumor yield too slight to permit any positive conclusion.

Experiment 9. (Chart 4, C). In this last experiment the order of the carcinogen used was reversed by giving 20-methylcholanthrene first and 4-nitroquinoline N-oxide second.

Mice were prepared by 10 applications of 20-methylcholanthrene and left without further treatment for 200 days. No tumor occurred in any of the mice during this period. A second treatment with 10 applications of 4-nitroquinoline N-oxide was given to 8 mice that were then surviving.

Four of these 8 mice developed papillomas within 20 days after the last application of 4-nitroquinoline N-oxide, and in 3 of them the papillomas turned into malignant growths. The fourth mouse with papilloma died early, before the benign growth became malignant. The tumors tended to appear earlier than in the case of 4-nitroquinoline N-oxide preceding 20-methylcholanthrene, although the final tumor yield did not significantly differ from the case of the reversed order of treatments.

The results of the above experiments adequately demonstrated that the effects of the first treatment with either one of the two carcinogens, though they failed to give rise to tumor formation, nevertheless persisted in submanifestational state for a long period of time, and were capable of summation with the effects of second treatment with the other carcinogen to rapidly produce tumors. It is of special interest to note that the carcinogenic response to the second treatment did not perceptibly differ when the second treatment followed immediately or 200 days after the first treatment. The number of mice is insufficient for a statistical treatment of the data, but the fact that the rest period of 200 days did not produce a noticeable reduction in the rate of tumor development may be taken to indicate, by extrapolation, that the submanifestational alteration of the cells consequent on the first treatment may be irreversible for the duration of the life of the animals.

Discussion

It was more than ten years ago when Druckrey and Küpfmüller (6) pronounced a principle of fundamental importance that cancerization results from the summation

of a multiple of irreversible alterations in the critical cell components (duplicants). By quantitative feeding experiments using 4-dimethylaminoazobenzene, they demonstrated that the final tumor yield depended on the total dose of the carcinogen rather than on daily doses into which the total dose was divided, while the time until the development of liver cancer decreased with increasing daily doses. In the dosage range of from 3 to 30 mg per day the induction time was inversely proportional to the daily dose, and the total dose necessary for cancer production was practically constant at about 1,000 mg per rat. This, as they pointed out, is totally unlike the usual pharmacological action, in which there is a certain "recovery phase" after each dose, so that the net effect depends on the individual daily dose instead of on the total dose. In carcinogenic action the total dose necessary to produce cancer does not become larger when given divided in smaller doses over a long period of time. From these facts Druckrey and Küpfmüller deduced that the effect of each small dose persists without loss and is added up, and, in short, that cancer production is due to the summation of many small effects, each of which is irreversible.*

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* Cramer and Stowell (7) showed that, with single doses of 0.1 mg each of 20-methylcholanthrene applied to the skin of mice, the total dose required to produce tumors in 100 percent of the mice varied according to the intervals between applications. Thus, application once every 2 weeks required 1.7 mg, application every 3 weeks 1.1 mg, application once every 4 weeks less than 0.9 mg, while if the application is performed thrice weekly the amounts of carcinogen necessary to produce the first tumor in a group of mice were as large as, or larger than, those required to produce tumors in 100 percent of the mice by more spaced applications. Experimental results of this nature would flatly contradict the summation theory, *if cancerization were the only effect of carcinogen*. We know, however, that a carcinogen produces many local changes accompanying but not necessarily related to cell cancerization.

The most relevant of the local changes in this connection may be non-specific cell injuries. Every time the carcinogen is applied to the skin, certain proportions of the cells *in loco* are injured, if not killed, so that the balance between cell cancerization and non-specific injury becomes a factor in determining the rate of tumor production. This balance is bound to swing more toward cell injury by too frequent applications of carcinogen, which may thus result in undue injuring or killing off of cells that are already in the process of cancerization. Clearly, no quantitative summation of carcinogenic effect can be expected under such circumstances.

Clayton and Baumann (8) fed a certain definite total amount of m-methyl-p-dimethylaminoazobenzene to rats, either continuosly or interrupted for periods of 2 to 12 weeks, and reported that the number of tumors decreased as the period of interruption was prolonged. Unfortunately, these authors compared the number of tumors, in all groups alike, at 29 weeks from the beginning of the azo dye feeding, regardless of the length of the interruption period, neglecting the natural delay in tumor production to be expected from the delay in the second part of the dye feeding. In order to render the experimental results valid, the period of observation for the interrupted feeding groups should have been extended by the length of interruption period, thus: 29+12=41 weeks for the group in which the dye feeding was interrupted for 12 weeks.

In discussing Druckrey's postulate, Shear (9) stated that with certain small doses of carcino-

Experiments reported in the present paper demonstrated with special clearness, and based on the summation of the effects of two chemically different carcinogens, that the cell change from normal to cancer may indeed be due to the summation of many submanifestational effects, which add up, possiblly one by one, until they reach the necessary total to manifest themselves by the production of a tumor.

It is now generally considered as almost self-evident that the essential part of the carcinogenic process must be some sort of genetic alteration in somatic cells, but it has not been fully realized that the cell genetic change in question consists of a multiple of self-perpetuating alterations. If each of these alterations is to be compared with a mutation, the process of cancerization may be likened to the summation of multiple mutations. One of the objections to the somatic mutation theory of carcinogenesis has been that mutation is a sudden, single-jump process, while carcinogenesis proceeds apparently gradually. The theory of summation of multiple mutation-like alterations may set aside this difficulty, and may explain why there are intergradations between completely normal cells and frankly cancerous cells. Cells complete their cancerization by degrees, it is true, but the changes progress by many small jumps, and each jump represents a persisting cell state toward the completion of cancerization. This situation is graphically shown in Chart 5.

The number of the necessary "mutations" (number of duplicants altered) involved in completing cell cancerization cannot be estimated even approximately. However, if we let Q and M represent the carcinogenic process accomplished by 10 applications of 4-nitroquinoline N-oxide and of 20-methylcholanthrene respectively, our experimental data concerning submanifestational doses prove that $Q<M$. Since Q must

genic hydrocarbons, the tumor yields do not increase after reaching a certain low percent, in spite of the "continued action" of the carcinogen, as long as the animals survived. Reference to his published data (confirmed by personal communication from Dr. Shear) shows that in his experiments the carcinogen was implanted in the form of pellets in subcutaneous tissue only once, and its "continued action" was assumed from the fact that the carcinogen can be recovered chemically unchanged from the pellets removed from the animals after many months. The possibility of the carcinogen in pellets, closely encapsulated and walled off *in situ*, being unavailable for action was not taken into consideration. It may be confidently predicted that even a very small dose of carcinogen, if it is capable of producing tumors in a low proportion of animals in a reasonable period of time, will increase the tumor yield later, if continued action is insured by repeated applications.

Finally, Rusch, Kline and Baumann (10), in experiments employing 20-methylcholanthrene or 9, 10-dimethyl-1, 2-benzanthracene in combination with ultraviolet light, noted the failure of summation of carcinogenic effects of these agents. In these instances, the difference in the target tissue, i.e., epithelium for the hydrocarbons and dermal connective tissue for ultraviolet light in mice (Grady, Blum and Kirby-Smith (11)) must be regarded as sufficient reason for throwing the experiments entirely off the mark.

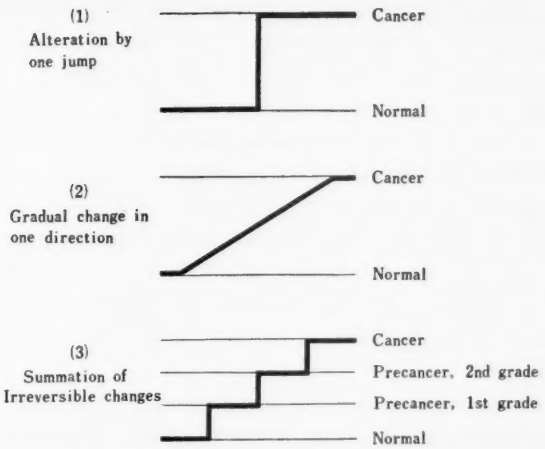

Chart 5. Diagrammatic representation of three concepts of the process of cell cancerization. (1) Alteration by one jumps. (2) Gradual change in one direction until the change becomes irreversible. (3) Summation of a number of alterations, each irreversible.

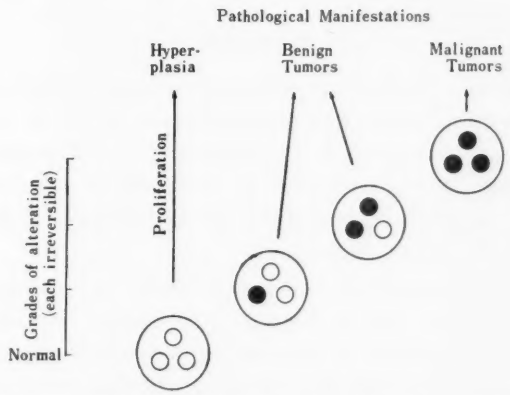

Chart 6. Diagram showing the summation of irreversible alterations under carcinogenic influence, and pathological manifestations of the proliferations of the cells which have undergone different grades of alteration.

involve the alteration of at least one duplicant, it follows that M effects the alteration of at least 2 duplicants. These considerations leads to the conclusion that the number of duplicants that must be altered in order to complete cancerization cannot be less than 3.

This conclusion implys that there are *at least three distinct grades of cancerousness*, which a normal cell undergoes during the process of its cancerization. Each of these grades represents an independent, irreversible alteration, which is capable of summation with further alterations that may occur later. If carcinogenic influence is withdrawn at any time during the process, the grade of cancerousness attained by that time is persistently retained by the cells and their progeny. The proliferation of the cells merely produces tissue growth corresponding to the grade of their alteration, normal cell producing hyperplasia, precancerous cells benign tumors, and fully cancerized cells malignant tumors. The ability to proliferate is a common attribute of all kinds of cells, and the different proliferative patterns reveal the different intrinsic nature of the cells as they proliferate. These relations are illustrated in Chart 6.

In postulating the summation theory of carcinogenesis, Druckrey (12) called attention to its obvious practical implication, referring to the possible danger from each unmanifested carcinogenic effect. Exposure to any carcinogenic influence induces submanifestational effect, which persists and remains for a long time capable of summation with similar effects received later and may be brought to tumor production. The results reported in the present paper demonstrated that the effects can summate even when they are due to carcinogens of quite different sorts.

Summary

Summation effect of two chemically unrelated carcinogens was investigated, using 4-nitroquinoline N-oxide and 20-methylcholanthrene. Doses of each of these two carcinogens that can be applied to the mouse skin without producing tumors (submanifestational doses) were first worked out, and then, in the main experiments, submanifestational dose of either one was given followed by that of the other and the rate of tumor production observed.

The results established that the submanifestational carcinogenic process started by 20-methylcholanthrene can be quantitatively brought to manifestation (tumor production) by 4-nitroquinoline N-oxide, and *vice versa*, showing that the effects of these two carcinogens, in spite of the striking chemical difference, are capable of summation, and suggesting that the immediate carcinogenic mechanism may be qualitatively identical for the two. It was also demonstrated that the submanifestational effect produced by the first treatment with either carcinogen persisted without any recognizable loss for at least 200 days, and was capable of summation with the effect of the second treatment, just as quantitatively as when the second treatment immediately followed the first.

These results definitely support the postulate that cancerization results from the summation of a multiple of alterations in the self-replicating cell subunits (duplicants),

each alteration being irreversible, and add an important fact that the alterations caused by a carcinogen of one type can summate with those that are due to another of different kind.

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[GANN, 51, 139~145; June, 1960]

SKIN CARCINOGENESIS WITH A SINGLE PAINTING OF 4-NITROQUINOLINE N-OXIDE

(Plates VIII and IX)

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INTRODUCTION

There have been a few reports in which cancers were successfully induced by a single application of one of the several kinds of potent carcinogenic polycyclic hydrocarbones (Mider and Morton[10], Low[9], Cramer and Stowell[2]). The lower the dose of the applied chemical agents, as it appeared, the lower was the induction rate of cancer and the longer the latent period. However it has been also known that the chemical agents so far applied in that type of experiments remained in loco for sometime, either intracellularly or extracellularly. This causes a difficult problem as to when the induction of intrinsic cell cancerization really occurs.

4-Nitroquinoline N-oxide, whose powerful carcinogenecity was recently reported by Nakahara and his colleagues[12], reacts on the contrary promptly in biological milieu[4,6] and the reaction products are known to be entirely impotent[13]. Thus, it must be assumed that if ever an application of 4-nitroquinoline N-oxide induced a cancer, the carcinogenic action should have taken effect almost immediately after the time of application. No retarded local effect is expected. Analysis of carcinogenesis has been made possible by the use of this new chemical.

The author reports in this paper a record of a series of experiments in which a single dose of different amounts of 4-nitroquinoline N-oxide was applied to the skin of the dd strain mice and the sequelae followed up to the 200th day of experiment.

EXPERIMENTAL METHODS

Two hundred and thirteen dd strain mice, each initially weighing 18-20 g and divided into four groups, were used in this experiment. The first group consisted of 10 females and 48 males, the second of 25 females and 23 males, the third of 50 males, and the fourth of 31 females and 26 males.

About 1/50 cc of 4-nitroquinoline N-oxide solution in acetone at four different concentrations were dropped on to the area of the skin of the back by means of a small glass pipette, the hairs having been pulled off 24 hours before the treatment.

The concentrations of the substance in the solutions used were 0.25%, 0.5%, 1.0%

and 1.9% (10^{-1}M), the absolute doses being 0.05 mg, 0.1 mg, 0.2 mg and 0.38 mg of the substance to the first, second, third and fourth group respectively.

All the animals surviving at the 200th day of experiment were killed and the painted skin region histologically examined. Some mice which dead before the term were also examined likewise. An excised sample of the skin was placed on a sheat of filter paper and fixed in ethanol-formalin (1 : 1) for 24 hours. Paraffine sections were stained with hematoxyline-eosin.

Observations

Early changes: At 24 hours, slightly edematous, partly seborrheic changes were observed in the treated areas of the skin. After 2 or 3 days, most parts of the treated areas became seborrheic and in some cases small erosions appeared. After 7 or 10 days seborrheic surfaces were covered with mixture of cornified and necrotic materials. In 20 to 40 days ring-formed or fleck-like depilated lesions were noticed in the treated areas. Tumors began to appear later somewhere in the depilated areas.

0.25%-group: In this group, started with total of 58 mice, 12 died within 50 days without showing any tumor growth. On the 90th day, two skin tumors were observed. One tumor appeared at the margin of the painted area. This was an over rice-grain sized tumor and found to be papillomatous on the 140th day when the animal died. Histological examination revealed a squamous cell carcinoma (Fig. 3). The other was papillomatous. However, this regressed spontaneously to almost complete disappearance after one month, and the mouse was histologically without recurrence on the 200th day.

0.5%-group: Three papillomas appeared in 3 mice of this group consisting of 48 mice initially. Two of 3 papillomas developed on the 45th day, which were over rice-grain size, while the third appeared on the 50th day. However, all these papillomas regressed spontaneously at about 100 days period. One of these 3 mice died on the 140th day free of papilloma. On the 200th day the other 2 mice still living were free from papilloma.

1.0%-group: In this group, started with 50 mice, five papillomas appeared in five mice during the period of 21 to 50 days of treatment, but two of them spontaneously regressed after one month and the mice survived without recurrence up to the 200th day. Two of the remaining 3 mice died on the 40th and 86th day with histological evidences of papilloma. However in the other mouse, a rice-grain-sized papilloma appeared at the margin of the painted skin area on the 21st day. The solitary papilloma broke off on the 50th day, but was soon after replaced by a new growth at the same site. On the 60th day the papillomatous mass has grown to a bean's size with cauliflower-like surface. Eventually this developed into a firm dome-shaped growth with broad base by the 130th day, apparently malignant. This

Table 1. Overall result of the experiment

Groups	Concentration of 4-NQO in acetone	Nomber of mice: Initial Number	Survivors after: 50 days: Total	50 days: No. with Ca.	50 days: No. with Pap.	100 days: Total	100 days: No. with Ca.	100 days: No. with Pap.	150 days: Total	150 days: No. with Ca.	150 days: No. with Pap.	200 days: Total	200 days: Na. with Ca.	200 days: Na. with Pap.	Number of animals with skin tumors: Carcicoma	Number of animals with skin tumors: Papilloma
I	0.25%	58 (10 ♀ 48 ♂)	46	0	0	46	1(4)	1	19	0	0	15	0	0	1	2(5)
II	0.5%	48 (25 ♀ 23 ♂)	32	0	3(1)	28	0	1	20	0	0	8	0	0	0	3
III	1.0%	50 ♂	50	0	5(2)	42	0	1	9	1	0	3	0	0	1	5(6)
IV	1.9%	57 (31 ♀ 26 ♂)	37	0	5(3)	18	0	1	3	0	0	3	0	0	0	5
Total		213 (66 ♀ 147 ♂)	165	0	13	134	1	4	51	1	0	29	0	0	2	15

(1) 2 of 3 showed spontaneous regression
(2) 2 of 5 died within 100th day and 2 show spontaneous regression.
(3) 3 of 5 show spontaneous regression.
(4) died on 140th day.
(5) one of two became carcinoma.
(6) one of 5 became carcinoma.

mouse died on the 162th day due to pneumonia. The mass was a squamous carcinoma (Figs. 1 and 2).

1.9%-group: In this group, 8 papillomas were found in 5 mice in a period of 21 to 30 days. Spontaneous regression of the lesions was observed in 3 mice after one month and the mice remained without recurrence up to the 200th day. The other two persisted until the 86th and the 146th day. One of them was a papilloma of over a rice-grain size when examined on the 86th day. The last mouse had 3 papillomas on the 21st day at the margin of the treated area, which grew rapidly and became conglomerated, forming a small finger-tip-sized mass. However, this papilloma broke off on the 70th day. The second growth was noticed on the 80th day at the same site. When examined on the 146th day, the mass had grown to a finger tip's size with cauliflower-like tumor surface. At the 200th day this was found histologically to be a benign squamous papilloma.

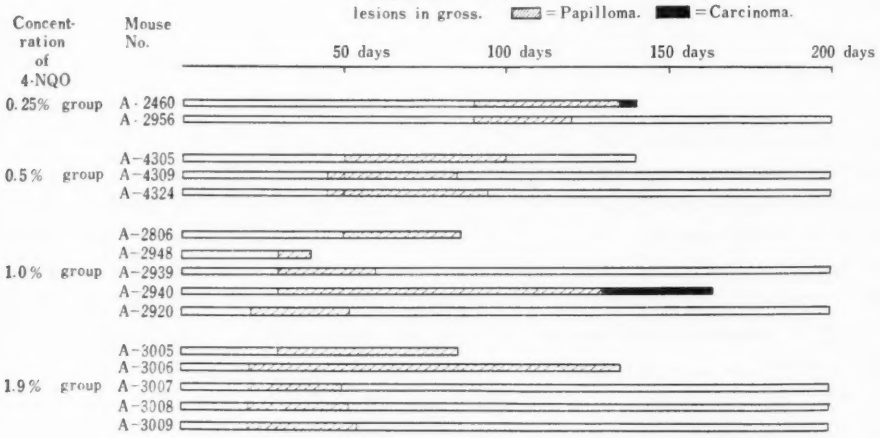
The above results are summarized in Table 1 and Chart 1.

Discussion

The present data clearly indicate that 4-nitroquinoline N-oxide is capable of producing epidermal carcinoma when painted on the mouse skin in acetone solution only once.

Although the experiment was started with a fairly large number of animals, many died due to infection of the colonies. As a whole the initial number of 213 of mice diminished to 29 survivors on the 200th experimental day. Two squamous carcinomas and 15 papillomas were observed sometimes during the 200 days period.

Chart 1 : Sequence of appearance and disappearance of tumorous lesions in gross. ▨ = Papilloma. ■ = Carcinoma.



Nine of the papillomas disappeared spontaneously, and 2 of them became malignant. Each of the carcinomas was preceded by apparently benign papillomatous proliferation. The rate of incidence of malignancy was about 7% of the survivors at the 200th day and less than 1% of the initial number of the animals. The different rates of survival in various dose-groups so confused the issue that no direct relation between the dose of 4-nitroquinoline N-oxide and incidence rate of skin carcinoma could be shown. However, as Chart 1 shows, it may be pointed out that there was a definite tendency for higher concentrations of the chemical to induce the papillomatous lesions in the painted areas earlier.

As compared with the previous reports on the epidermal carcinogenesis following a single application of polycyclic hydrocarbons, the present data reveal rather low carcinogenecity of 4-nitroquinoline N-oxide. Mider and Morton[10] in 1939 succeeded to produce skin carcinoma in the C 57 brown mice in 7% and 4% respectively in their 2 series of experiments in which they painted mouse skin only once with a 0.5% solution of methylcholanthrene. Similar experiment by Low[9] (1941) with a single application of 9,10-dimethyl 1,2-benzanthracene yieled 8 carcinomas in 10 mice. Many of the carcinomas developed outside of the area painted with the chemical agent. Cramer and Stowell[2] reported in 1943 the development of skin carcinoma in 6 of 14 Swiss mice after a single dosage of 0.3 mg of methylcholanthrene. Thus it has been revealed that with the highly potent carcinogenic polycyclic hydrocarbons as high as 80% of experimental animals can be induced to develope malignancies with a single dose under the most adequate experimental conditions. Although in one group (1% group) of the author's series, one of the 3 mice surviving on the 200th day developed carcinoma, the overall incidence rate appears much lower than that in the previous reports on hydrocarbons.

There are evidences showing that in case the hydrocarbons were used the agents remain in loco in active forms for some length of time. Beck and Peacock[1] found the mouse skin to remain fluorescent for about 4 days after a single application of benzpyrene-acetone. Hamperl, Graffi and Langer[5] reported the fluorescence of uncornified layers of the mouse epidermis after a single application of benzpyrene-benzene to persist unchanged for 48 hours before it began to fade away. Heidelberger and Weiss[7] reported that the radioactivity after a single application of 3,4-benzpyrene-5-C^{14} decreases, first rapidly and then slowly during the first few days. Even two to three weeks after the application, the skin areas maintained radioactivity though at a rate less than 10% of the original value. Carcinogenic hydrocarbons are known to become protein-bound in the tissue milieu (Heidelberger). Lorenz and Stewart[8] emulsified 20-methylcholanthrene in horse serum and testified that the emulsion was potent.

Thus, it appears reasonable to say that the carcinogenic polycyclic hydrocarbons

exert prolonged actions to the tissues even if they are given in a single dose and that they can cause carcinogenic effects in a way similar to the case of repeated applications of the same agents.

In contrast, Nakahara and Fukuoka[13] found a mixture of 4-nitroquinoline N-oxide and human serum was completely inactive after standing at room temperature for thirty minutes. In the biological milieu this substance reacts promptly with tissue sulfhydryl groups (Endo[4] and Hayashi[6]) and loses its potencies. As has been pointed out by Nakahara, the carcinogenic activity of 4-nitroquinoline N-oxide may thus be displayed only promptly after the application but never later.

If 4-nitroquinoline N-oxide can ever induce a cancer with a single application to the tissue, the cancer should have been initiated exactly there and then. The present data are very interesting because they have brought out the evidence that such can really occur although at a low induction rate.

There have been many opinions con and contra to the two major hypotheses in the steps of cancerization. In the two-phase theory of Berenblum, Rous and Rusch the phase of initiation of cancer is interpreted as a specific type of sudden at random jumps from normal to abnormal but a qualitatively different "promoting effect" is assumed to be needed to induce the "initiated" cells to proliferate to form tumors. Druckrey and Küpfmüller[3], on the contrary, suggested in 1948 that summatian of a number of irreversible alterations are necessary for cancerization. This postulate was strongly supported by Nakahara and Fukuoka[14], based on the summation of carcinogenic actions of two chemically unrelated carcinogens, 4-nitroquinoline N-oxide and 20-methylcholanthrene.

Although the demonstration that cell cancerization can, though infrequently, be accomplished by a single hit of the force of a chemical reaction can by extenuation be interpreted by either of the two hypotheses or theories above mentioned, it seems difficult to conceive of a single chemical reaction simultaneously playing the two qualitatively different roles of "initiation" and "promotoin." It can of course be insinuated that the absence of adequate "promoting effect" is responsible for the low rate of cancer production. On the other hand, a single hit by a force can easily be imagined to produce a multitude of alterations in the cell, the efficacy depending no doubt on the magnitude and nature of the force in relation to the target cell components. However, it is not the purpose of the present paper to decide the merits of the two theories, for which purpose the data presented are inadequate.

Summary

4-Nitroquinoline N-oxide was painted only once on the skin of 213 dd strain mice at four different concentrations in acetone, and the subsequent development of skin lesions followed for 200 days. Fifteen papillomas were induced, of which nine

disappeared spontaneously and four persisted, and two of these latter eventually became squamous cell carcinoma.

The known prompt chemical reaction of this carcinogen in biological milieu and the non-carcinogenicity of the entire reaction products suggest that even a single hit of the force of a chemical reaction is at least at times capable of inducing all the intrinsic alterations in the cell that are necessary for cancer production.

The author wishes to express his hearty thanks to Dr. Waro Nakahara and to Dr. Kunio Oota. Thanks are also due to Dr. Hideya Endo, Dr. Tsuneo Baba and to Dr. Yuzo Hayashi for their helpful suggestions.

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Explanation of Plates VIII and IX

Fig. 1. Gross appearance of A-2940 on 130 days after treatment. The tumor is seen on the margin of the painted area.

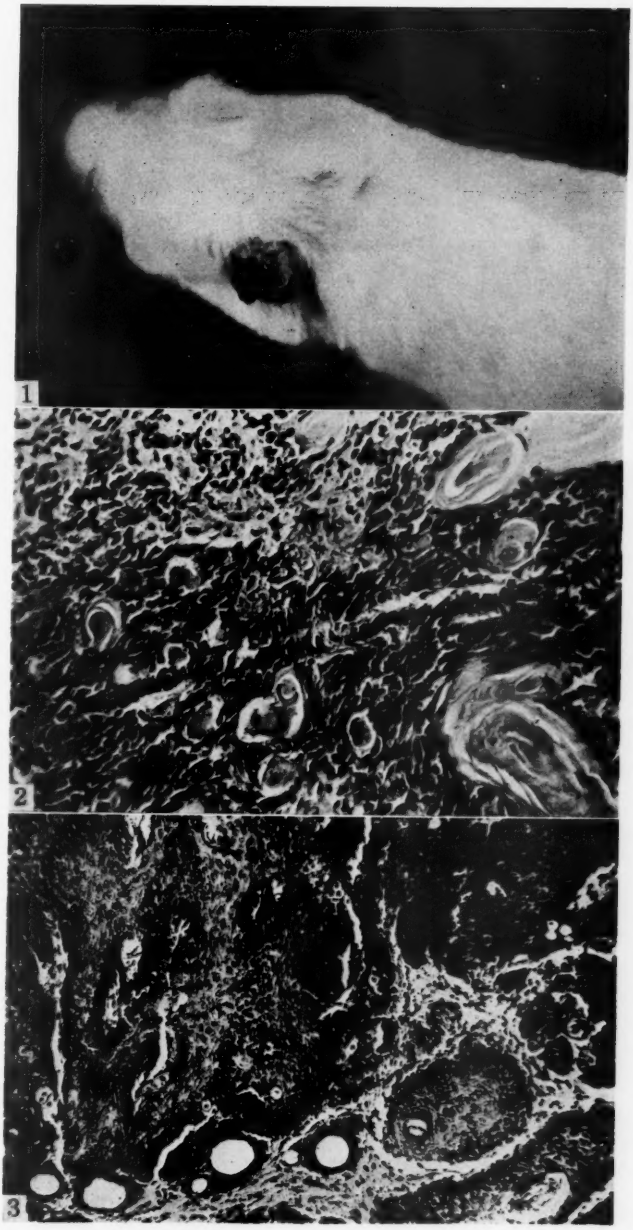
Fig. 2. Microscopic picture of the tumor of fig. 1. Rather analpastic squamous cell carcimoma. H. and E. ×200

Fig. 3. A squamous cell carcinoma (A-2460) which appeared 140th days after a single application. H. & E. ×70

Fig. 4. Gross appearance of A-3006 on 136th day.

Fig. 5. Histological picture of the tumor of fig. 4, showing benign squamous cell papilloma. H. & E. ×5

Fig. 6. A typical case of papilloma of A-2806, showing a central crater of keratin mass. H. & E. ×70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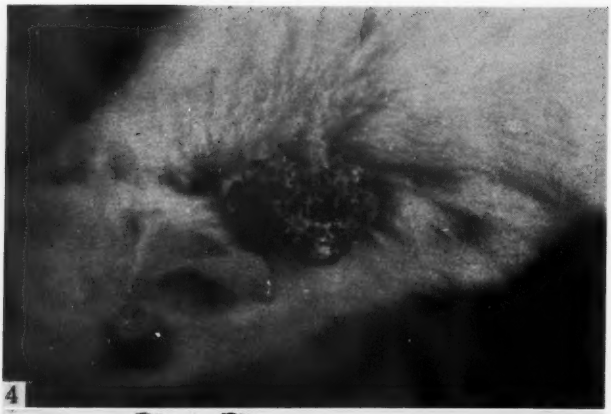

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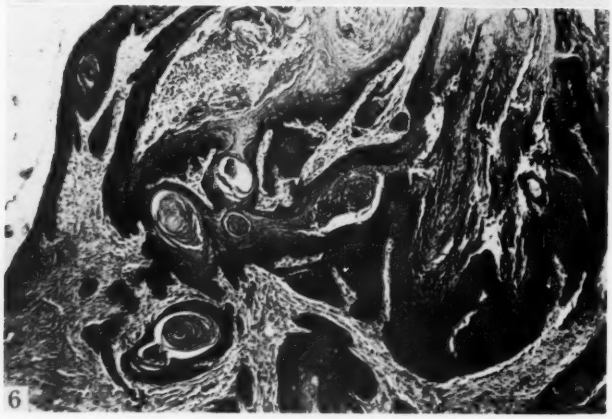

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[GANN, 51, 147～152 ; June, 1960]

AUTORADIOGRAPHIC AND ELECTRONMICROSCOPIC STUDY OF CHEMICALLY INDUCED FIBROSARCOMA IN MICE

(Plates X—XIII)

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INTRODUCTION

It has been known that the ground substance of the connective tissue is chemically characterized by a high content of sulphomucopolysaccharide. A series of experiments[4,5,8,9], particularly using S^{35}-labeled sulphate *in vivo* and *in vitro*, has shown that the synthesis of mucopolysaccharides was an intracellular process. As reviewed by Boström[3], the activity of mucopolysaccharide synthesis in the mesodermal cells is strongly influenced by various physiological and pathological conditions, including aging of cells, metabolic or hormonal disorders, and inflammation.

The present study was attempted in order to find out some difference in the activity of sulphomucopolysaccharide synthesis between the chemically induced fibrosarcoma and granulation. The tissues, excised after the administration of S^{35}-labeled sulphate, were examined comparatively with autoradiography and also with electronmicroscopy.

MATERIAL AND METHOD

40 male mice of dd-strain, initialy weighing around 18 g, were used. They were subcutaneously injected with 0.05 cc of 0.5% solution of 20-methylcholanthrene in olive oil. The injection was repeated 5 times, once a week at the same site of the lumbosacral region. The total amount of 20-methylcholanthrene administered was 1.25 mg per mouse.

18 mice survived 9 weeks after the last injection. They were given intraperitoneal injection of aquous solution of S^{35}-labeled sodium sulphate in a dose of 1 μc per gram body weight, with 0.2 mg of non-labeled sodium sulphate as carrier, and were killed 24 hours thereafter. The treated parts were excised fresh and fixed in ethanol-formalin solution (1 : 1) for 24 hours. The paraffin sections were cut at 6μ. Hematoxyline-eosin, azan, Masson, PAS and alcian blue staining were used for histological examination.

For the autoradiography stripping type, Fuji. ET. 2E, 15μ, was used. The time of exposure was 4 weeks at 4°C.

Electronmicroscopy : Small fragments of the excised lesions were fixed fresh in

veronal buffered 1.0% osmic acid solution. The ultra-thin sections were examined with JEM-type of electronmicroscope.

Results

A. Pathohistological findings of the subcutaneous lesions:

Of the 18 mice, five had subcutaneous tumors, ranging from a bean to a thumb-tip in size. All these tumors revealed themselves to be spindle cell sarcoma consistent with the diagnosis of fibrosarcoma. The cellular components were seen closely packed. Limited areas of necrosis were present more or less in every case.

Two of the other animals had rice-grain sized epidemal tumors: one of which was a benign papilloma and the other was an anaplastic spindle cell carcinoma.

In the other 11 animals, the treated parts of the subcutis showed moderate indurations, which consisted of focal proliferation of fibroblasts. Vascular proliferation with occasional capillary dilatation and accumulation of a variety of round cells were associated more or less conspicuously in every case. These were thought to be consistent with benign granulation tissue reaction to the chemical injuries.

The histochemical results were listed in Table 1.

B. Autoradiographic findings:

1. Most part of the intact loose subcutaneous tissue showed a moderate diffuse blackening. The papillary layer of the dermis was higher in radioactivity than the

Table 1. Comparison of autoradiographic, histochemical and electronmicroscopic findings.

	Intact subcutaneous tissue	Granulation tissue	Fibrosarcoma
The uptake of S^{35}-labelled sulphate(Intensity of blackening)	+～++	+++	+～++
Histochemistry of AMP* (PAS and Alcian blue)	+～++	++～+++	+～++
Intercellular spaces	narrow	wide	narrow
mature collagen-fiber	+++	−	−
immature collagen-fiber	−	++	−
Cytoplasmic organellae			
endoplasmic reticulums (ER)	++～−	+++	+++
Palade granules	+	+～++	+++
density of ER contents	−	−～++	+++
mitochondria	+	++	+++
Golgi apparatus	n.r.	n.r.	n.r.
microbodies	+	+++	+++
osmophilic bodies	+	++	+
cytoplasmic inclusions	−	++	−

*AMP=acid mucopolysaccharide n.r.=non-remarkable change.

other part. The bundles of mature collagen fibers were negative for radioactivity.

2. A much higher degree of blackening was noticed in the granulation tissue than in the intact subcutis. The highest degree of blackening appeared in the proliferative part. The fibrous and hyalinized portions of the glanulation were low in radioactivity.

3. The tissue of fibrosarcoma exhibited a much less blackening than the granulation. The degree of the blackening was nearly equal to those in the papillary layer of the dermis. The necrotic focus was negative in the radioactivity.

C. Electronmicroscopic findings:

1) The subcutaneous tissue of non-treated mice consisted almost exclusively of mature collagen bundles and fibrocytes. The cytoplasmic organellae were non-remarkable. The endoplasmic reticulums were moderately developed and their lumina were electron lucid. The intercellular spaces were free of immature collagen or amorphous substance.

2) The granulation tissues, induced by the treatment with methylcholanthrene, consisted of many proliferating fibroblasts scattered in the midst of amorphous ground substance rich in delicate immature collagen fibrils. The fibroblasts were full of cytoplasmic organellae. The endoplasmic reticulums which were much dilated, were filled with homogenous substance of moderate electron density. This was associated with increase in Palade granules. The microbodies were found in moderate number. Often irregularly demarcated, finely granulated inclusions of higher density were observable in the cytoplasm. Some regenerative myocytes appeared at the marginal areas.

3) The fibrosarcomas were composed of tumor cells closed crowded together. Their nuclei were large and pleomorphic, and provided usually with a single nucleolus. The mitochondria were abundant and varying in size, some of them containing dense particles. The endoplasmic reticulums were dilated and filled with elctron-dense homogenous substance, quite similar to those in the case of the granulation tissue. The Palade granules were much increased in number. There was, however, no inclusion body in the cytoplasm. The Golgi apparatus appeared non-remarkable. The intercellular spaces were very narrow and practically revealed no ground substance. The collagen fibers were absent, mature or immature.

Discussion

A noticeable fact obtained in the present study was a quantitative difference in the tissue incorporation of radioactive-sulphate between the fibrosarcoma and granulation. While a prominently high fixation of S^{35}-sulphate was noticed in the granulation, the fibrosarcoma exhibited a rather slight radioactivity. These findings nearly correspond with the data by Boström et al.[2]. According to them, benzpyrene-

induced rat fibrosarcomas were less active in the S^{35}-sulphate fixation than the normal dermal tissue. In the present observation, however, the S^{35}-sulphate fixation of fibrosarcoma was almost equal to or slightly higher than those of the resting subcutaneous tissue.

Layton et al.[7], using a tissue culture technique, found a higher S^{35}-sulphate fixation in the embryonic tissue than in the abult. He also reported[8] a greater retention of sulphate in granulation. Curran et al.[4] have confirmed this fact in the autoradiographic study of quartz-granuloma, and showed the highest incorporation of S^{35}-sulphate in the fibroblastic zone. In the present observation, the highest fixation of S^{35}-sulphate was noticed also in the part of the granulation in the proliferative stage. Fibrous parts were invariably low in radioactivity in the same specimens.

Although the above findings indicate, as the previous authors suggested, a close parallelism between the proliferative activity and S^{35}-sulphate fixation in a series of fibroblastic tissues, this appears not to be true in conjunction with malignan detrivatives of the same tissue. As it has been clearly shown by Dziewiatkowski[6] (1951) and Boström (1952)[1], sulphur-S^{35} given as sulphate ion is fixed only in the sulphomucopolysaccharide of the tissue. The present data suggest low synthetic activity of the substance in the fibrosarcoma despite its high rate of proliferation as revealed in the growth and mitotic frequency.

The electronmicroscopic findings were partly consistent with the above view. In fibrosarcoma the tumor cells were extremely closely packed, the intercellular spaces were narrow, and there was no apparent sign of fibril production in the mass. Hewever, as an intracytoplasmic event, there could be no direct clue as to the synthesis of the mucopolysaccharides in the electronmicroscopic findings. The endoplasmic reticulums were dilated equally in the fibrosarcoma and in the granulation tissue and were filled with electron-dense homogenous substance. There was no indication that the endoplasmic reticulums were directly concerned with synthesis of the mucopolysaccharides. Meyer[10] postulated that mucopolysaccharides act as templates or organizers in the development of the architecture of the organized fibrous elements of the connective tissue. Accoring to this hypothesis, the abundance of immature collagen fibrils in the granulation tissues may be consistent with the active synthesis of sulphomucopolysaccharide as revealed in the autoradiographs.

Summary

1. A comparative autoradiographic and electronmicroscopic study was performed on the fibrosarcoma and benign granulation tissue induced by subcutaneous injection of 20-methylcholanthrene to mice.

2. Although a definite parallelism was noticed between the proliferative activity and S-^{35}sulphate fixation in the benign fibroblastic tissue, this appeared not true in

conjunction with the malignant tissue.

3. The abundance of immature collagen fibrils in the granulation tissue was assumed to be consistent with the active synthesis of sulphomucopolysaccharide as revealed in the autoradiographs.

4. Electronmicroscopy revealed no direct evidence of sulphomucopolysaccharide production in the tissue.

Our hearty thanks are due to Dr. Kunio Oota, Cancer Institute, Tokyo, and Pathology Department of Tokyo Medical and Dental College, under whose guidance the present study was made. Our thanks are also due to Mr. Hiroyasu Miyamoto, Mr. Ken Aoki, Miss Tomoe Fujiwara and to Miss Minako Niijima for their indispensable technical help.

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Explanation of Figures (Plates X—XIII)

Figs. 1, 2, 3, 4, 5 & 6: A fibrosarcoma, 1: H & E, 2: Autoradiograph (8×). Most part of the tissue is moderately blackened. Figs. 4, 5, & 6 are higher magnification (200×) of the parts (A, B & C) of Fig. 1. The necrotic foci (B, Fig. 5) are negative in radioactivity.

Figs. 7 & 8: One area of a papilloma (100×). The stroma is moderately blackened. The epithelial part is almost negative in radioactivity.

Figs. 9 & 10: A subcutaneous lesion (15×). The part of granulation (upper right) exhibits a high fixation of S^{35}-sulphate. The sarcoma (lower part) is low in radioactivity.

Figs. 11 & 12: One area of granulation tissue (100×). A prominent blackening is noticed in the proliferative part.

Figs. 13 & 14: One area of granulation tissue (200×). Hyalinized portion (lower left) is almost negative in radioactivity.

Fig. 15: Electronmicroscopic picture of untreated subcutaneous connective tissue of mouse (5,500×). A fibrocytes embedded among mature collagen fiber bundles. The intercellular spaces are very narrow.

Fig. 16: A part of a fibrocyte shown in Fig. 15 (37,500×). The endoplasmic reticulums are small and their contents electron-lucent.

Fig. 17: Electronmicrogroph of a part of the granulation tissue, consisting of proliferating fibroblasts & wide intercellular spaces with ground substances and immature collagen fibrils (5,500×).

Fig. 18: Detail of a fibroblast in Fig. 17 (granulation tissue) (37,500×). The endoplasmic reticulums are much dilated are filled with homogenous substance.

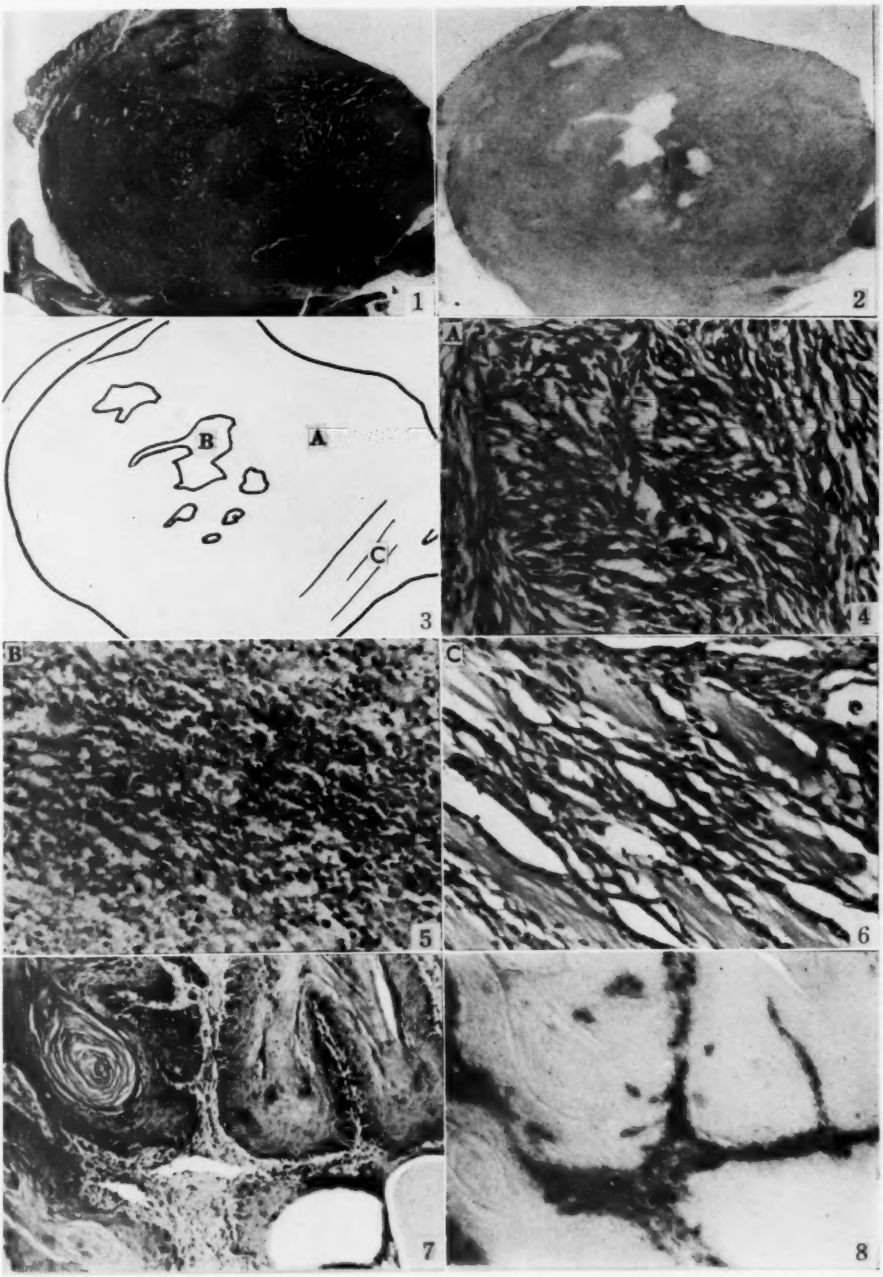
Fig. 19: Detail of a fibroblast in Fig. 17 (granulation tissue) (28,250×). A fibroblast rich in mitochonchondria and osmophilic bodies.

Fig. 20: Electronmicrograph of fibrosarcoma (5,500×). The fibrosarcoma cells are rich in organellae. The intercellular spaces are quite narrow. No demonstrable collagen.

Fig. 21: Detail of a fibrosarcoma cell (37,500×). The endoplasmic reticulums are moderately dilated and filled with electron-dense homogenous substance.

Fig. 22: Detail of a fibrosarcoma cell (37,500). Abundant mitochondria, one of which containing irregular shaped dense particles, and osmophilic bodies.

N=nucleus	G=Golgi apparatus
er=endoplasmic reticulum	mt=mitochondria
Os=osmophilic body	d=dense particle in mitochondria
imf=immature collagen fibril	mf=mature collagen fiber
Fc=fibrocyte	Fb=fibroblast
Fs=fibrosarcoma cell	Is=intercellular space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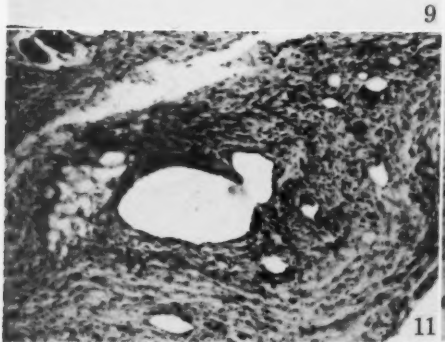
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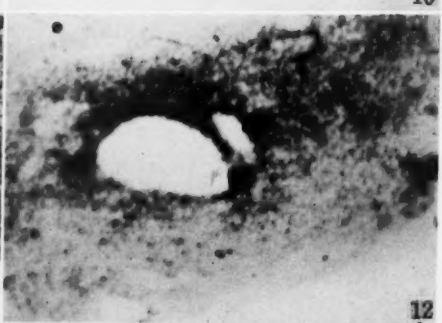
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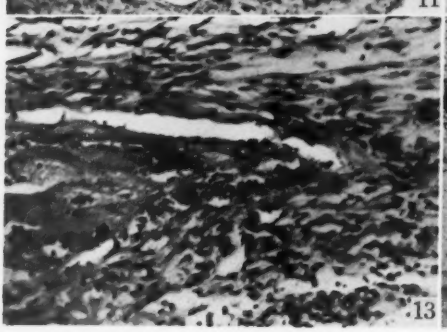
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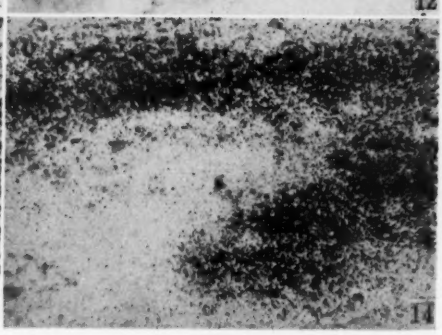
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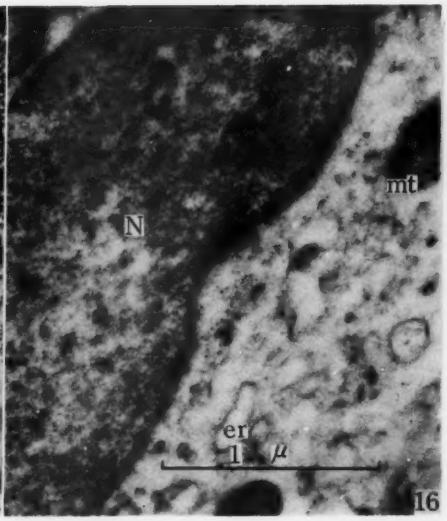
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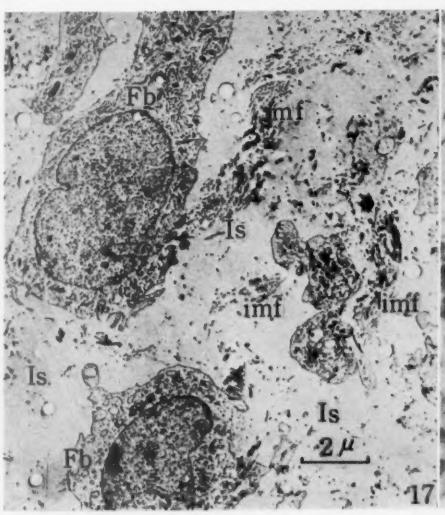
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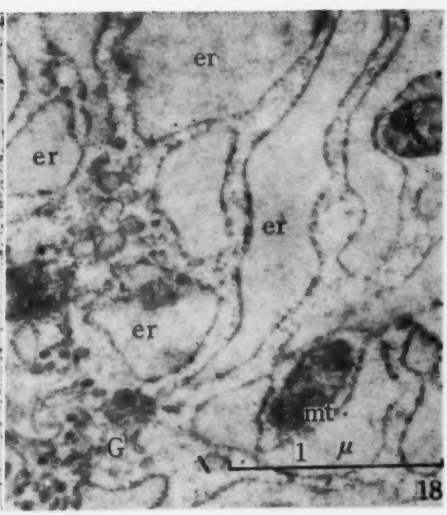
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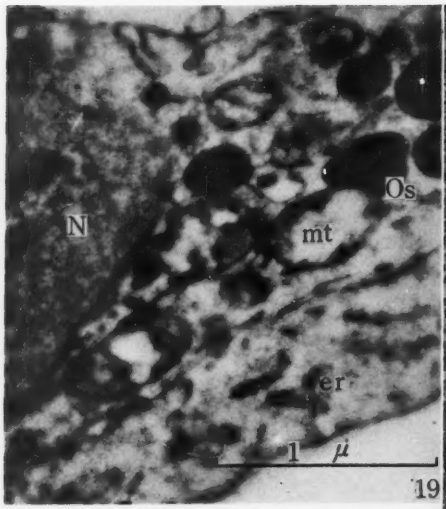

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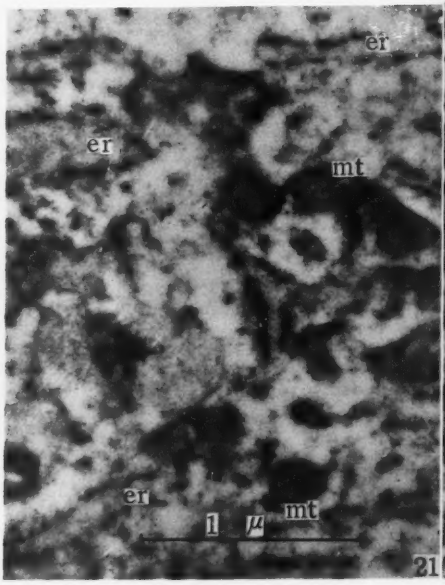

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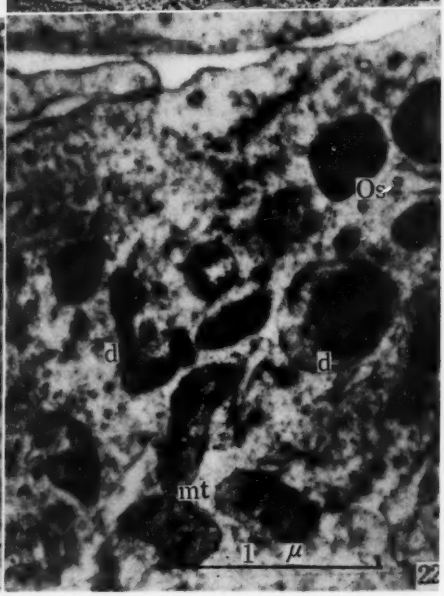

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[GANN, 51, 153~158; June, 1960]

CHANGES OF SOME FLAVIN ENZYMES IN THE LIVER OF RAT DURING DAB CARCINOGENESIS. II

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Changes of D-amino acid oxidase, xanthine oxidase and succinic dehydrogenase in the liver of rats which were fed DAB for about one month were reported in a previous paper (1), in which it was demonstrated that these flavin enzymes were affected in different ways by DAB feeding.

This paper shows the effects of DAB feeding on the activities of DPNH-cytochrome C reductase, TPNH-cytochrome C reductase, Straub diaphorase, and lipoflavoprotein (2).

Materials and Methods

DPNH was prepared by the chemical method of Lehninger (3), using commercial DPN. TPNH was prepared by the enzymatic method of Evans and Nason (4), using TPN prepared by the method of Horecker and Kornberg (5).

Cytochrome C was prepared from beef heart by the method of Okunuki et al. (6). The percentage of autooxidizable cytochrome C was found to be 4.6 by the method of Tsou (7). Ferricytochrome C was obtained by acidifying a solution of cytochrome C with 0.01 N-HCl to pH 3, and then neutralizing it with an equivalent amount of alkali. FAD and 2,6-dichlorophenol indophenol used were commercial products.

Animals. Albino rats weighing 150-200 g were fed for about one month on DAB-coated rice (8). The control rats were fed on rice coated only with olive oil.

The animals which were used for the assay were decapitated and exsanguinated. The livers were removed and placed in cracked ice immediately. The weighed livers were homogenized with a glass homogenizer in a suitable volume of isotonic KCl solution, and centrifuged at 3,000 r.p.m. for 5 minutes to remove the cell debris.

Assay methods. DPNH-cytochrome C reductase was assayed according to the method of Mahler et al. (9).

The standard reaction mixture contained the following final concentrations in a total volume of 3.0 ml: 0.01 M-phosphate buffer pH 7.4, 10^{-3}M potassium cyanide, 280μg of DPNH (determined from the optical density at 340 mμ), 0.8 mg of ferricytochrome C

The abbreviations used are: DPN—diphosphopyridine nucleotide; DPNH—reduced diphosphopyridine nucleotide; TPN—triphosphopyridine nucleotide; TPNH—reduced triphosphopyridine nucleotide; DAB—p-dimethylaminoazobenzene; FAD—flavin adenine dinucleotide.

(determined from the optical density at 550 mμ) and 0.01 ml of the supernatant of 2 percent homogenate.

In the control cuvette, DPNH was omitted. After the enzyme was added, changes of optical density, measured at 550 mμ were determined every 30 seconds, using the Shimadzu QB-type spectrophotometer.

TPNH-cytochrome C reductase was assayed essentially according to the same method as DPNH-cytochrome C reductase (9), except that 6 percent homogenate was used as an enzyme solution because of its lower activity in rat liver. Straub diaphorase was assayed according to the same method as DPNH-cytochrome C reductase, except that 2,6-dichlorophenol indophenol was used instead of cytochrome C (9). The reaction velocity was determined by measuring the decrease of optical density at 600 mμ at 30-second intervals, after the addition of 2 percent homogenate. Lipoflavoprotein was measured by the same method as Straub diaphorase except that ferricyanide was used instead of 2,6-dichlorophenol indophenol. The reaction velocity was determined by measuring the decrease of optical density, measured at 410 mμ, aftar the addition of 2 percent homogenate (2, 10).

Table 1. DPNH-Cytochrome C Reductase in the Liver of Normal and DAB-fed Rats.
(Expressed by μ moles of cytochrome C reduced/min/g of wet liver)

Exp. No.	Normal Rats	Exp. No.	DAB-fed Rats
1	6.9	7	4.2
2	7.9	8	4.6
4	8.6	9	4.6
5	9.6	12	4.7
		13	6.3
		14	6.3

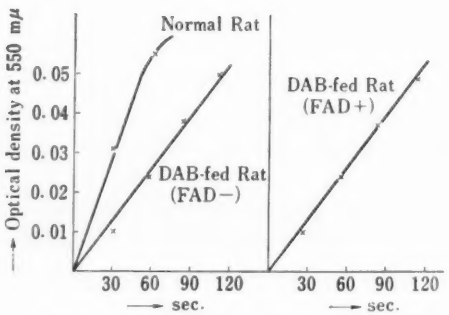

Fig. 1 DPNH-Cytochrome C Reductase in the Liver of Normal and DAB-fed Rats.

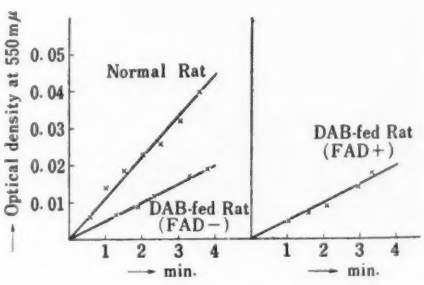

Fig. 2 TPNH-Cytochrome C Reductase in the Liver of Normal and DAB-fed Rats.

Results

1) **DPNH-Cytochrome C Reductase.** The activity of this enzyme in the liver of normal and DAB-fed rats is shown in Table 1. In DAB-fed rat liver 4.2–6.3 μmoles of cytochrome C were reduced, while 6.9–9.6 μmoles of cytochrome C were reduced in normal rat liver. As shown in Figure 1, FAD, added *in vitro*, could not restore the activity of the enzyme in DAB-fed rat liver. Thus, the protein part of this enzyme seems to be altered by DAB feeding.

2) **TPNH-Cytochrome C Reductase.** The activities of this enzyme in normal and DAB-fed animals are demonstrated in Figure 2 and Table 2. As shown in the table, 0.24–0.63 μmoles of cytochrome C were reduced in DAB-fed rat liver, while 0.52–0.68 μ moles of cytochrome C were reduced in normal rat liver. It is evident that the activity of TPNH-cytochrome C reductase is about one-tenth lower than that of DPNH-cytochrome C reductase in normal rat liver. The *in vitro* additon of FAD did not restore the reduced activity of this enzyme.

3) **Diaphorase.** Since the diaphorase activities were measured in crude tissue preparations, the values given in Table 3 and Figure 3 are the sum of the activities of DPNH-cytochrome C reductase and diaphorase. Under these experimental conditions, however, the activity of DPNH-cytochrome C reductase need not be taken into account, since the activities of diaphorase (Table 3) are much higher than that

Table 2. TPNH-Cytochrome C Reductase in the Liver of Normal and DAB-fed Rats.
(Expressed by μ moles of cytochrome C reduced/min/g of wet liver)

Exp. No.	Normal Rats	Exp. No.	DAB-fed Rats
25	0.52	30	0.24
26	0.58	31	0.26
27	0.58	32	0.29
29	0.68	35	0.31
		36	0.37
		38	0.42
		39	0.63
		40	0.63

Table 3. Diaphorase in the Liver of Normal and DAB-fed Rats.
(Expressed by μ moles of 2, 6-dichlorophenol indophenol reduced/min/g of wet liver)

Exp. No.	Normal Rats	Exp. No.	DAB-fed Rats
15	45	19	42
16	56	20	52
17	61	22	54
		24	69

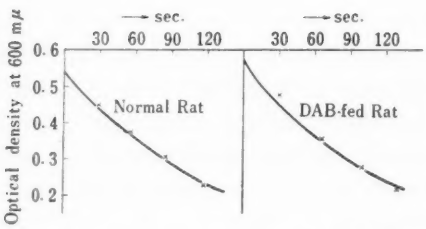

Fig. 3 Diaphorase in the Liver of Normal and DAB-fed Rats.

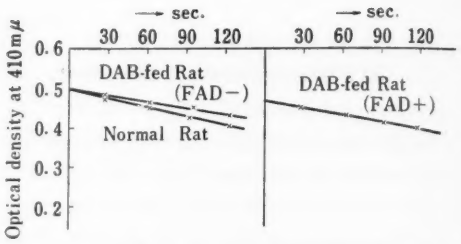

Fig. 4 DPNH Dehydrogenation Activity by Ferricyanide of Normal and DAB-fed Rats' Liver.

of DPNH-cytochrome C reductase (Table 1). The values given in Table 3 must be doubled when the activities of these two enzymes are to be compared on the basis of a one-electron acceptor, since the cytochrome C is a one-electron acceptor and the 2,6-dichlorophenol indophenol is a two-electron acceptor. Therefore, the diaphorase activity is high enough to neglect the cytochrome C reductase activity. It may therefore be concluded that the diaphorase activity is not affected by DAB feeding.

4) **Lipoflavoprotein.** Ziegler et al. recently isolated a lipoflavoprotein from beef heart mitochondria. They showed that the flavoprotein can be converted either to diaphorase or DPNH-cytochrome C reductase, depending upon the experimental method used. It is therefore doubtful whether the diaphorase and DPNH-cytochrome C reductase are present as separate entities in the mitochondria.

The lipoflavoprotein is capable of utilizing ferricyanide as an electron acceptor. Table 4 and Figure 4 show that the activity of the DPNH-ferricyanide system of the DAB-fed rat liver is slightly lower than normal.

Table 4. DPNH Dehydrogenation Activities by Ferricyanide of Normal and DAB-fed Rats' Liver

(Expressed by μ moles of ferricyanide reduced per min. per g of wet liver)

Exp. No.	Normal Rats	Exp. No.	DAB-fed Rats
41	120	46	90
43	120	47	96
44	126	49	99
45	132	52	102
		53	102
		55	106

Discussion

Recent histological studies (13, 14) have revealed that DAB and its related compounds induce cholangiocarcinoma, but not hepatoma. It is improper, therefore, to

use normal liver as the control tissue for a long time during azo-dye carcinogenesis.

However, these data demonstrated the changes of liver flavin enzymes after one month of DAB feeding. The histologic studies of these DAB-affected livers after one month's feeding showed about a ten percent increase in cholangiol cells. Hara, in our laboratory, found that after up to three months of DAB feeding, C-1-fructokinase of normal liver is not replaced by C-6-fructokinase, which is characteristic of cancer tissue; but, after five months, the liver fructokinase was transformed into a cancerous type (15).

If the present experiments are accepted as showing tissue responses to the ingested dye, these data may be of some significance in the study of carcinogenesis.

Throughout this series, profound changes were seen among liver flavin enzymes in the cases of D-amino acid oxidase and succinic dehydrogenase. The protein part of D-amino acid oxidase was almost completely destroyed. On the other hand, the apoenzyme of succinic dehydrogenase was intact while its coenzyme was almost completely lost.

As to the oxidation of reduced pyridine nucleotides by the flavin enzymes, the electron transfer to a two-electron acceptor is not affected, but the transfer to one-electron acceptors is slightly but definitely affected.

Summary

1) Rats were fed on DAB-coated rice for about one month. DPNH dehydrogenation activities of the liver were determined in the presence of several electron acceptors.

2) DPNH-cytochrome C reductase in the liver of rats fed DAB was lower than normal.

3) TPNH-cytochrome C reductase was about one-tenth of that of DPNH-cytochrome C reductase, and was definitely reduced by DAB feeding.

4) Diaphorase activity was about fifteen times higher than DPNH-cytochrome C reductase in normal rat liver and not reduced by DAB feeding.

5) Lipoflavoprotein activity in the liver of rats fed DAB was slightly lower than normal.

6) The *in vitro* addition of FAD did not restore the reduced activities of cytochrome reductases or lipoflavoprotein.

The authors wish to thank Prof. S. Mizuhara for his helpful suggestion and Miss R. Wakamatsu for her technical assistance. This investigation was aided in part by a grant from the Ministry of Education.

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STUDIES ON ZINC-METABOLISM OF TUMOR. II. BEHAVIORS OF ^{65}Zn- AND ^{203}Hg-HEMATOPORPHYRINS INJECTED INTO EHRLICH ASCITES TUMOR-BEARING MOUSE*

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INTRODUCTION

When the ^{65}Zn-glycine complex was injected into the Ehrlich ascites tumor-bearing mouse, ^{65}Zn was found by the authors to be accumulated into the tumor cells and to be maintained for a fairly long time in the course of proliferation of the cells[1]. These phenomena are very interesting in relation to Fujii's hypothesis that the zinc plays an important role in the mitosis of cells[2]. From the fact that the ^{14}C-labeled glycine itself was accumulated into the tumor cells[3], however, the question arises whether ^{65}Zn can be incorporated or not into the cells in only the form of glycine complex, that is to say, whether ^{65}Zn has or not the affinity to the tumor cells in any other chemical form. On the other hand, the accumulation of the injected porphyrin was found in the tumor tissues by Auler[4] and Figge *et al.*[5] Considering this fact, it is expected that the tumor cells might take up more of the zinc attached to the hematoporphyrin ring in the same way as the zinc-glycine complex. In the present paper, it is shown that the behavior of ^{65}Zn-hematoporphyrin injected into the tumor-bearing mouse is contrary to the above expectation and a factor which controls the uptake of zinc (generally, of metal) by the tumor cells is discussed by comparing the behaviors of ^{65}Zn-glycine complex and ^{65}Zn- and ^{203}Hg-hematoporphyrins.

EXPERIMENTAL METHODS

Animals and Tumor: The Ehrlich ascites tumor was transplanted in the inbred

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ddN strain of male and female mice, each of which has about 20 g of body weight. Transplantations were always done by intraperitoneal inoculation with about 0.1 ml of seven day-old tumor ascites fluids.

Preparations of Isotope Solutions:

(a) ^{65}Zn-hematoporphyrin solution: 50 mg of ^{65}Zn-hematoporphyrin-Na_2 was dissolved in 20 ml of distilled water. This aq. solution contained 2.2 μc/ml of radioactivity.

(b) ^{203}Hg-hematoporphyrin solution: 40 mg of ^{203}Hg-hematoporphyrin-Na_2 was dissolved in 20 ml of distilled water. The radioactivity of this aq. solution was estimated to be 7.39 μc/ml.

Injections of Isotope Solutions: The ^{65}Zn- or ^{203}Hg-hematoporphyrin solution was injected intraperitoneally or subcutaneously on the back of the mouse on the fifth day after the transplantation of tumor. As the control for the experiment using ^{65}Zn-hematoporphyrin, the injections of the ^{65}Zn-glycine complex solution containing 2.2μc/ml of radioactivity were made under the same conditions as the ^{65}Zn-hematoporphyrin was injected.

Preparations of Samples: The animals were killed by cervical dislocations on the first, third and sixth days after the injection of the isotope solutions. The ascites fluids collected from the abdominal cavity were immediately centrifuged and the supernatant, namely, the ascites plasma was pooled as the sample for determination of radioactivity. The tumor cells as the sample were obtained from the recovered cells by washing with the hypertonic sucrose solution and then by recentrifuging. If the recovered cells contained erythrocyte, they were hemolyzed previous to the washing procedure. On the other hand, the liver, kidney and spleen were removed by blunt-dissection. In the case of ^{65}Zn-hematoporphyrin, the above samples were digested with 5 ml of conc. sulfuric acid added a small amount of both potassium and copper sulfates and then their radioactivities were measured. In the case of ^{203}Hg-hematoporphyrin, the loss of ^{203}Hg in the course of wet-ashing with sulfuric acid forced us to adopt the following procedure. Previous to the digestion with conc. sulfuric acid, the ascites plasma, the tumor cells suspended in water, and the liver homogenate were provided as the samples for determinations of radioactivities.

Determination of Radioactivity and Total Nitrogen: As described in a previous paper[1], the radioactivity was measured with a scintillation counter and the total nitrogen was determined by the micro-Kjeldahl method.

Experimental Results

Behaviors of ^{65}Zn-Hematoporphyrin: Fig. 1 shows the radioactivity in counts per min. per 100 mg of nitrogen found in each sample after the subcutaneous injection of 0.2 ml of the ^{65}Zn-hematoporphyrin solution on the back of the mouse which is bearing

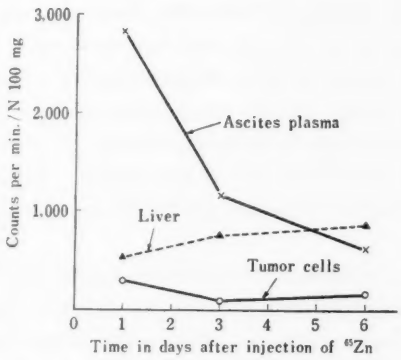

Fig. 1 The radioactivities recovered in Ehrlich ascites tumor cells, its ascites plasma, and the liver of the tumor-bearing mouse, which received subcutaneously 0.44μc of ^{65}Zn-hematoporphyrin on the fifth day after the inoculation of tumor.

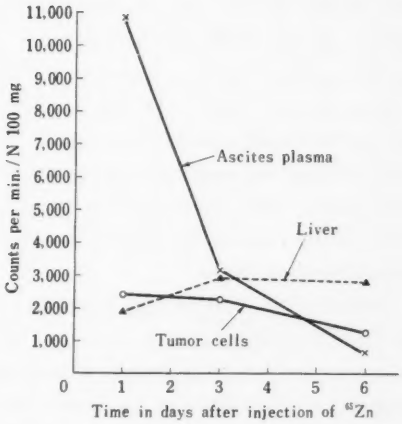

Fig. 2 The radioactivities recovered in Ehrlich ascites tumor cells, its ascites plasma, and the liver of the tumor-bearing mouse, which received intraperitoneally 1.10μc of ^{65}Znhematoporphyrin on the fifth day after the inoculation of tumor.

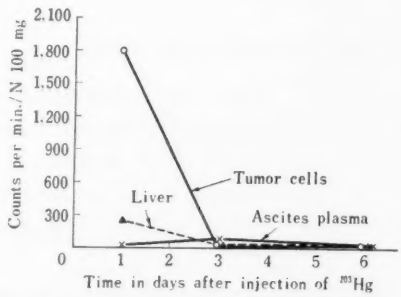

Fig. 3 The radioactivities recovered in Ehrlich ascites tumor cells, its ascites plasma, and the liver of the tumor-bearing mouse, which received subcutaneously 1.48μc of ^{203}Hg-hematoporphyrin on the fifth day after the inoculation of tumor.

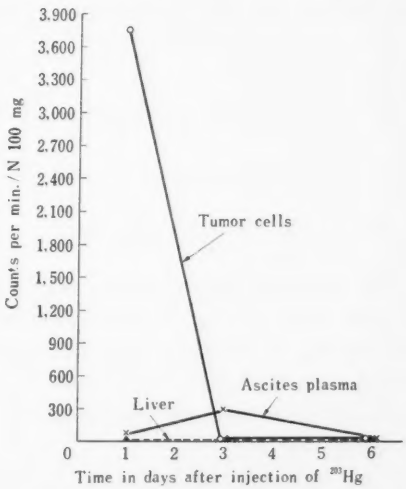

Fig. 4 The radioactivities recovered in Ehrlich ascites tumor cells, its ascites plasma, and the liver of the tumor-bearing mouse, which received intraperitoneally 1.48μc of ^{203}Hg-hematoporphyrin on the fifth day after the inoculation of tumor.

five day-old tumor. On the first day, the radioactivity was largely recovered in the ascites plasma, from which then fairly rapidly disappeared with the lapse of days. It was hardly, however, found in the tumor cells between the first and sixth days.

The results are shown in Fig. 2 where each mouse received intraperitoneally 0.5ml of the ^{65}Zn-hematoporphyrin solution. In the ascites plasma, the radioactivity largely remained on the first day and then fairly rapidly decreased in the course of days. The tumor cells, liver and the other tissues tested have low radioactivities. While the activities slowly increase in the liver and spleen, those of the kidney and tumor cells slowly decrease.

Behaviors of ^{203}Hg-Hematoporphyrin: In both cases of the subcutaneous and intraperitoneal injections of 0.2 ml of the ^{203}Hg-hematoporphyrin solution, the radioactivity in the tumor cells was largely recovered on the first day but diminished to nearly zero on the third day. In the liver and ascites plasma, the radioactivities were very low or nearly zero through the period from the first to sixth days. These results are shown in Figs. 3 and 4.

Discussions

It was shown in a previous paper[1] that the difference between the apparent and actual excretion coefficients of the radioactivity denoted by $\bar{\alpha}$ and α, respectively, is equal to the specific rate coefficient of the increase of total nitrogen, μ, that is,

$$\alpha - \bar{\alpha} = \mu \qquad (1)$$

where

$$\alpha = -\frac{1}{S}\frac{dS}{dt} \qquad (S = \text{c.p.m./100 mg of nitrogen}),$$

$$\bar{\alpha} = -\frac{1}{Z^*}\frac{dZ^*}{dt} \qquad (Z^* = \text{total c.p.m./whole tissue}),$$

$$\mu = \frac{1}{N}\frac{dN}{dt} \qquad (N = \text{total nitrogen/whole tissue}).$$

These coefficients are generally functions of time, t. When the radioactivity, S, and the total nitrogen, N, after the injections of ^{65}Zn-hematoporphyrin are rearranged in semi-logarithmic scale, the coefficients α and μ are found to be constant and independent on time as shown in Figs. 5 to 8. The values of α and μ calculated from the above data are summarized in Tables 1 and 2 with the value of $\bar{\alpha}$ estimated by using Eqn. (1).

From the results of control experiments using ^{65}Zn-glycine complex, the coefficients α, $\bar{\alpha}$ and μ are calculated in the same way and summarized in Tables 3 and 4.

It can be clearly seen in Tables 3 and 4 that the ^{65}Zn of zinc-glycine complex is accumulating in the tumor cells ($\bar{\alpha} = -0.461$ and -1.524) in spite of the fact that the radioactivity is on the apparent decrease ($\alpha = 0.264$) in the case of intraperitoneal injection. (See Fig. 3 in a previous paper[1].)

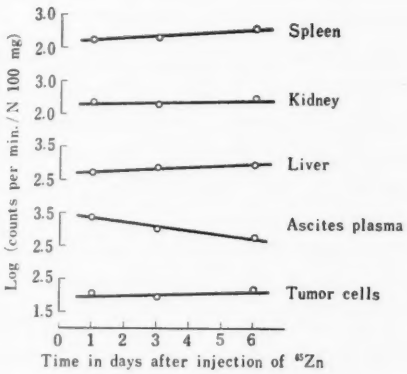

Fig. 5 The apparent excretion of the radioactivities in the tumor cells, its ascites plasma and various tissues of Ehrlich ascites tumor-bearing mouse, which received subcutaneously 0.44μc of ^{65}Zn-hematoporphyrin on the fifth day after the inoculation of tumor.

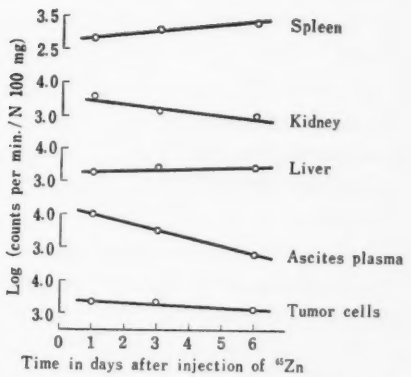

Fig. 7 The apparent excretions of the radioactivities in the tumor cells, its ascites plasma and various tissues of Ehrlich ascites tumor-bearing mouse, which received intraperitoneally 1.10μc of the ^{65}Zn-hematoporphyrin on the fifth day after the inoculation of tumor.

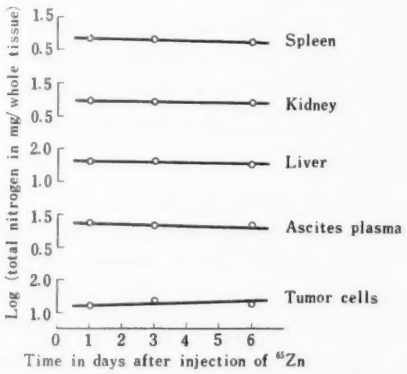

Fig. 6 The increases of the total nitrogen of the tumor cells, its ascites plasma and various tissues of Ehrlich ascites tumor-bearing mouse, which received subcutaneously 0.44μc of ^{65}Zn-hematoporphyrin on the fifth day after the inoculation of tumor.

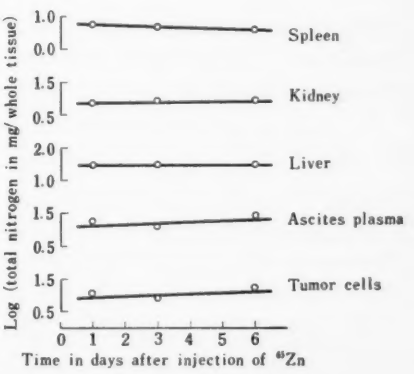

Fig. 8 The increases of the total nitrogen of the tumor cells, its ascites plasma and various tissues of Ehrlich ascites tumor-bearing mouse, which received intraperitoneally 1.10μc of ^{65}Zn-hematoporphyrin on the fifth day after the inoculation of tumor.

Table 1. The specific rate of increase of total nitrogen and the apparent and actual excretion coefficients after the subcutaneous injection of ^{65}Zn-hematoporphyrin.

	Tumor cells	Ascites plasma	Liver	Kidney	Spleen
Specific rate of increase of N, μ	0.001	−0.048	−0.050	−0.022	−0.068
Apparent excretion coefficient, α	−0.056	0.300	−0.088	−0.061	−0.176
Actual excretion coefficient, $\bar{\alpha}$	−0.057	0.348	−0.038	−0.039	−0.108

Table 2. The specific rate of increase of total nitrogen and the apparent and actual excretion coefficients after the intraperitoneal injection of ^{65}Zn-hematoporphyrin.

	Tumor cells	Ascites plasma	Liver	Kidney	Spleen
Specific rate of increase of N, μ	0.075	0.078	−0.003	0.006	−0.109
Apparent excretion coefficient, α	0.124	0.551	−0.088	0.162	−0.214
Actual excretion coefficient, $\bar{\alpha}$	0.049	0.473	−0.085	0.156	−0.105

Table 3. The specific rate of increase of total nitrogen and the apparent and actual excretion coefficients after the subcutaneous injection of the ^{65}Zn-glycine complex.

	Tumor cells	Ascites plasma*	Liver	Kidney	Spleen
Specific rate of increase of N, μ	0.024	—	−0.062	−0.033	−0.151
Apparent excretion coefficient, α	−0.437	—	0.263	0.142	0.104
Actual excretion coefficient, $\bar{\alpha}$	−0.461	—	0.325	0.175	0.255

*The coefficients can not be obtained from the data with respect to the ascites plasma, because the graph of log S or log N *versus* time does not become a straight line.

Table 4. The specific rate of increase of total nitrogen and the apparent and actual excretion coefficients after the intraperitoneal injection of the ^{65}Zn-glycine complex.

	Tumor cells	Ascites plasma	Liver	Kidney	Spleen
Specific rate of increase of N, μ	1.788	0.235	−0.038	−0.024	0.004
Apparent excretion coefficient, α	0.264	0.073	0.136	0.071	0.160
Actual excretion coefficient, $\bar{\alpha}$	−1.524	−0.162	0.174	0.095	0.156

On the other hand, the values of $\bar{\alpha}$ in Tables 1 and 2 show that the actual excretion of the radioactivity from the ascites plasma goes on at fairly high rate ($\bar{\alpha}$=0.348 and 0.473) and that the activity of tumor cells does not much decrease or increase ($\bar{\alpha}$= −0.057 and 0.049).

It has been observed by Ely and Batt[6] that the hematoporphyrin does not go within the living ascites tumor cells and can be adsorbed on the leucocyte, dead cells or cell

debris. Considering their observations, a low radioactivity of the tumor cells shown in Figs. 1 and 2 may be due to (1) the ^{65}Zn-hematoporphyrin adsorbed on the tumor cells, which cannot be removed by washing, or to (2) the ^{65}Zn liberated from the hematoporphyrin ring by hydrolysis or by substitution with the other metals and then incorporated into the tumor cells. According to the classification of metallo-porphins by Barnes and Dorough,[7] the zinc-hematoporphyrin belongs to the group of small divalent metal complex. In this group, the metal (zinc) atom fits in the space surrounded by the four nitrogen atoms of porphin ring. The chemical bonds between zinc and nitrogen atoms, therefore, would be resistant to hydrolysis and replacement by the other metals, though, these covalent bonds have partly ionic nature. This fact disapproves the second possibility where the ^{65}Zn incorporates into the tumor cells after the liberation from the porphyrin ring. The first possibility is also supported by the fact that the ^{65}Zn-content of the tumor cells shows both inconsistent tendencies of increase and decrease and their values are nearly zero ($\bar{\alpha}=-0.057$ and 0.049), contary to the case of ^{65}Zn-glycine complex. It may be thus concluded that ^{65}Zn-hematoporphyrin does not accumulated in the tumor cells. The temporary high radioactivity of the ascites plasma is, however, considered to be due to the ^{65}Zn-hematoporphyrin from the same point of view. This high radioactivity fairly rapidly decreases with the biological half-value period of a day or two. On the other hand, Figge *et al.*[5] observed that the injected zinc-hematoporphyrin was accumulated in the tumor tissues of mice which are bearing the methylcholanthren-induced sarcoma and was maintained for a long time between ten and fourteen days. This difference of the behaviors of zinc-hematoporphyrin may be attributed to the difference of conditions in the ascites plasma and the intercellular fluids.

The behaviors of ^{203}Hg-hematoporphyrin can also be explained from the same viewpoint. In the group of large divalent metal complex, which the mercury-hematoporphyrin belongs to, the size of metal atom is too large to fit easily into the space surrounded by the four nitrogen atoms. The mercury atom, then, juts out from the intramolecular space and are held mostly by coulombic forces with the nitrogen atoms. They are therefore unstable against the hydrolysis or the replacement reaction. This nature suggests that the mercury atom might be liberated from the hematoporphyrin ring and taken up by the tumor cells. This expectation is realized in Figs. 3 and 4. Contrary to the case of ^{65}Zn-hematoporphyrin, the high radioactivity on the first day is referred to the ^{203}Hg taken up by the tumor cells. The damage of tumor cells by ^{203}Hg and their disintegrations may bring about the subsequent marked decrease of radioactivity accompanied with a decrease of the volume of packed tumor cells. In any case, it may be safely said that the uptake of metal (zinc or mercury) by the tumor cells is partly decided by the nature of the chemical bond, namely, the type of bond, its strength, and the stereochemistry or steric factor, *etc.*,

between the metal atom and its ligands in the complex compound.

An inhibition of the growth of the tumor can be also seen when the ^{65}Zn-hematoporphyrin solution was injected. As described in a previous paper[1], the μ is considered to be equal to the specific rate of multiplication of cells provided that the nitrogen content per cell is constant throughout the life of the cell. In both cases where the injections were made subcutaneously and intraperitoneally, the value of μ of the tumor cells after the injection of ^{65}Zn-hematoporphyrin is only a twenty-fourth part of that after the injection of ^{65}Zn-glycine complex as shown in Table 5.

Table 5. The anti-tumor action of ^{65}Zn-hematoporphyrin, assuming that the specific rate of increase of total nitrogen is equal to the specific rate of multiplication of cells.

Specific rate of increase of N, μ, in the case of	Subcutaneous injection 0.44μc	Intraperitoneal injection 1.10μc
^{65}Zn-hematoporphyrin	0.001	0.075
^{65}Zn-glycin complex	0.024	1.788
Inhibition effect*	1/24	1/24

*The inhibition effect is defined as the ratio of the value of μ in the case of ^{65}Zn-hematoporphyrin to that of ^{65}Zn-glycine complex.

This result leads us to an idea of the " radio-dynamic action ", that is, the damage of the living cells by the cooperation of hematoporphyrin with the radiation from ^{65}Zn atom. This idea is supported by the experiment that the glycolytic metabolism of Ehrlich ascites tumor cells can be inhibited by the irradiation of X-rays in the presence of hematoporphyrin[8]. Still more investigations are, however, necessary for the establishment of a role of the radiodynamic action in the anti-tumor mechanism.

Summary

The behaviors of ^{65}Zn- and ^{203}Hg-hematoporphyrins injected into Ehrlich ascites tumor-bearing mice were studied in comparison with that of the ^{65}Zn-glycine complex and the following results were obtained.

1. In both cases of the subcutaneous and intraperitoneal injections of ^{65}Zn-hematoporphyrin, the high radioactvity was found in the ascites plasma on the first day after the injections and then disappeared at fairly high rate with the lapse of days. A low or no radioactivity recovered in the ascites tumor cells did not so much vary in the course of days.

2. In both cases of the subcutaneous and intraperitoneal injections of ^{203}Hg-hematoporphyrin, the high radioactivity was found in the tumor cells on the first day and then rapidly reduced to nearly zero on the third day after the injections. The

ascites plasma and the liver contained no radioactivity during the period from the first to sixth days.

3. These behaviors can be understood from the point of view that the uptake of metal (zinc or mercury) by the tumor cells may be controlled by the natute of the chemical bond between the metal atom and its ligands in the complex compound.

Acknowledgement

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[GANN, 51, 169~178; June, 1960]

ULTRAVIOLET MICROSCOPIC STUDIES ON THE LIVING YOSHIDA SARCOMA CELLS

(Plates XIV~XVI)

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The cytological characteristics of Yoshida sarcoma have been widely studied with fixed preparations, and in recent years the morphological features in its living states have been studied with the phase contrast microscope (29, 64).

In the present study the living Yoshida sarcoma cells, both in resting and mitotic states, are observed with the aid of UV microscope. Since it is well known that both nucleic acids and proteins are constituents of the cell with the absorption maximum at wavelength of 260 mμ and 280 mμ respectively, the distribution of nucleic acids and proteins within the living cells can be determined as that of absorbing substances by the photomicrograph taken in these wavelengths.

Material and Methods

Tumor ascites, 2 to 8 days after transplantation, were diluted with Gey's balanced salts solution. The tumor cells were observed at the wavelength of 546 mμ at 36–37°C, and subsequently UV photographs were taken at 265 mμ, 280 mμ and 312 mμ. No UV irradiation was allowed except when photograph was taken.

Monochromator (Bausch & Lomb) and the reflecting type objective and condencer (both Bausch & Lomb) were used. The accuracy of the wavelength was adjusted to less than ±5 mμ by closing the slit of monochromator. The magnification on the film was 500× (or sometimes 300×), and exposure time at 265 mμ was 15 seconds, and total UV exposure was 30.5 seconds. The emulsion used for photomicrograph was Gevaert Scientia 37C50 film, which was developed in G-201 at 20°C for 8 minutes. This film is ultra sensitive to UV between 250 mμ and 300 mμ.

Beside UV microscopical investigations, phase contrast microscopical observations and examinations of the tumor cells supravitally stained with neutral red and with azur B (34) were also performed.

Results

Resting cells: Homogenous and strong absorption is noted in the cytoplasm except the juxtanuclear area, which is non-absorbing. This non-absorbing region

is varied as to its location according to the form, number and position of the nucleus (Figs. 1-7), and is not clearly defined from the surrounding cytoplasma. This region in photographs at 280 mμ is less conspicuous than at 265 mμ. Occasionally UV absorbing globules indefinite in size are found in this area. By phase contrast microscopic observations, many fine granules are found in this region, which caused indistinct characterization (Fig. 1c). Some of them (called "Golgi granules" by some authors) are stainable supravitally with neutral red, and they are usually in the rosett arrangement (Fig. 8). Using azur B, the ground substances of this region are weakly positive, while granules in this region are strongly positive. With subsequent UV photographs of these stained cells this region is quite obscure at both 265 mμ and 280 mμ.

Occasionally, in the cytolpasm except for juxtanuclear non-absorbing region, are seen round or thread-like granules which seem to be strongly absorptive (Fig. 2). In some cells, still larger granules (Fig. 3) or vesicles (Fig. 7), 1-10 or more in number, are noted. It is well justified from the photographs of the same cells taken at 312 mμ that they have no specific absorption. However, one cannot decide their nature only by the UV microscope.

Prolonged observation causes cytoplasmic blebbing or protrusion to take place around the cell bodies. The occurrence of this degenerating process is not limited to the cells exposed to UV light, but all of the cells on the same preparation degenerate in the same fashion. By degenerating process, the cytoplasm loses its original absorptivity, and finally the differences between the cytoplasm and the background becomes obscure. On such an occasion, however, nuclei remain in original structure, which look like nacked nuclei.

Nuclei are varied in shape and number as already reported (29). Nucleic materials, however, are always far less absorptive at both 265 mμ and 280 mμ. The nuclear membrane is found clearly on any UV photographs. In the nuclear sap obscure network, which is more absorptive, is usually noted. It may correspond to the chromatin threads or fragments of chromosomes in the interphase. Some of them are in contact with the nucleolus (Figs. 1, 2).

Nucleolus is clearly noted as a extremely dense structure. It is usually single, but varies in shape. Its position is not definite either. Nucleolus usually consists of two elements; the heavily absorptive one is dot- or rod-like or takes the form of horse-shoe or circle. The other is structureless, less absorptive, and fills the space between the former (Fig. 2). However, in some nucleoli only homogeneous and strong absorption at 265 mμ is noted. Even in such a case the two elements can be frequently distinguishable from each other at 280 mμ (Fig. 9).

Mitotic Cells

Prophase: As chromosomes become strongly absorptive both at 265 mμ and 280 mμ, the non-chromosomal nuclear substances (44) gradually lose the absorptivity. At the early stage of prophase there is still a nuclear membrane separating the juxtanuclear non-absorbing region and non-chromosomal nuclear substances from each other, but by the dissolution of the membrane both are conglomerated to a single non-absorbing region. This region is not clearly limited from the surrounding cytoplasm which has become less absorbing than that of interphase cells (Figs. 11-13). The heavily absorptive element of nucleolus forms some of the chromosomes together with the chromosomal fragments which touch the nucleoli. While the less absorptive element gradually loses its absorption until it completely disintegrates. As a result, nucleolar structure disappears.

Metaphase: With the complete arrangement of chromosomes on the equatorial plane, juxtanuclear non-absorbing region is converted to spindle region together with the non-chromosomal nuclear substances. (Figs. 14, 18). The holizontal view reveals star-shaped arrangement of the chromosomes in the spindle region (Fig. 15-17).

Anaphase: This phase takes only few minutes as already reported (27). It is within the spindle region that the daughter chromosomes migrate toward opposite poles. The interzonal region—that is between the two daughter chromosome groups—shows a very strong absorption at 265 mμ which is clearly distinguishable from the adjacent cytoplasm. At the early stage of anaphase the absorption at 265 mμ of this region is almost the same as that of the chromosomes, while the absorption at 280 mμ of the former is a little weaker than that of the latter (Fig. 20). As mitosis progresses, however, this region loses its absorptivity until there is no difference between the interzonal region and the adjacent cytoplasm. The pole-ward region which was less absorptive in metaphase cannot be distinguished from the adjacent cytoplasm. Therefore, at the late stage of anaphase, only the daughter chromosomes can be seen in the homogeneously and weakly absorptive cell bodies.

Telophase: After the chromosome movement, the cleavage furrow begins in the middle of the cell body, the shape of which has extended both ways toward the poles to become oval. At the same time the area around each daughter chromosome group loses its absorbability. When mitosis ends, the cell body is divided into daughter cells, in each of which the daughter nucleus had been reconstructed. The process of telophase is just contrary to that of prophase; a part of the non-absorptive region surrounding daughter chromosomes becomes the juxtanuclear non-absorbing region later, and the rest of the region, together with daughter chromosomes, becomes a daughter nucleus (Fig. 19). The less absorptive substances appear around a certain part of the chromosomes, which become the heavily absorp-

tive element of the nucleolus later. Then the nucleolus is reconstructed.

Mitotic Abnormalities: It has been reported by many authors that Yoshida sarcoma has mitotic abnormalities, especially the high frequency of binucleated cell formation. The process of this type takes place as follows UV micrographically: during anaphase the interzonal region gradually loses its absorptivity until it becomes practically non-absorptive. This is quite different from normal process (see anaphase). At the same time the regions around the two daughter chromosome groups lose the absorption as in normal telophase. Consequently, these and the interzonal regions conglomerate into the shape of a dumb-bell. A cell in this state will never divide, no matter whether a furrow may take place in the middle of the cell body or not. While the two daughter nuclei at both poles are reconstructed, the interzonal region changes into the juxtanuclear non-absorbing region between the two nuclei (Fig. 21).

Discussion

It is well known that morphological or functional change is caused in the cell by UV irradiation, and most effective wavelength to give severe change is in the area of maximum absorption (250–280 mμ) of both nucleic acids and proteins (8, 43, 58). To cause such a change in the cell a definite amount of UV irradiation is necessary, which varies according to the kind of cells (9, 15, 17, 58, 60). In the living Yoshida sarcoma cells there was no change detected after taking up to ten photographs at 265 mμ. Besides, it was found that mitotic cells seemed to be free from irradiation injury when they were photographed ten times (with intervals), though it cannot be proved that absolutely no injury is induced in the cells. Therefore in the experiment in this report was made a rule to take only 3 photographs of the same cell, each at 265 mμ, 280 mμ and 312 mμ, so that the cells observed might be practically free from UV irradiation damage.

The findings at wavelength of 265 mμ and 280 mμ show not only the specific absorption of nucleic acids and proteins respectively, but also nonspecific light loss which may be caused by scattering or reflecting. According to Walker (61), nonspecific light loss in the UV microscopic observation of living cells is very little in comparison with that of fixed cells. However, in order to obtain the pure specific absorption, photographs taken at wavelength of 312 mμ were used for comparison.

Golgi region and the juxtanuclear non-absorbing region: Many observations of Golgi apparatus in living cells with the phase contrast microscope have verified the concept that the apparatus is presented in the living state, and not an artefact (5, 13, 14, 22, 53, 54, 55, 57). This component of living cells consists of many granules and filaments packed in a definite cytoplasmic area which can be distinguished from the other part of the cytoplasm. This area is generally called Golgi region. That

the principal elements of Golgi region are cytochemically lipids and polysaccharides (neutral mucopoly-saccharides) has been reported (7, 11, 46, 57). Lasfargues and Fine (34), who studied the living fibroblasts stained supravitally with azur B, demonstrated that the Golgi region was specifically positive with strongly stained granules.

The juxtanulear non-absorbing region has very little UV absorbing materials in comparison with the surrounding cytoplasm and also is supravital stain positive. These facts suggest that this region corresponds to the Golgi region. The Golgi apparatus of Yoshida sarcoma has never been sufficiently appreciated electron-microscopically (26, 30, 62) except in the observation made by Yamada* who has clearly noted it in the niche of the nucleus (unpublished). According to him, this area is occupied by many fine granules which are different in electron density from other parts of cytoplasma. These fine granules can be regarded as Golgi ground-substance (1, 45) with Golgi lamellae and vesicles (or granules) in the central part of this area. This area also corresponds in location to this region.

Golgi region during mitosis: At present time, little is known about the role in the cell function of Golgi apparatus. Some authors (4, 18, 24, 63) reported that the centrioles were presented in the Golgi zone of resting cells. According to Amano (4), the central bodies are responsible for the Golgi body formation in interphase, and they make spindle fibers in the mitotic cells. Hanaoka (23) pointed out that Golgi body of the plasmacyte easily undergoes vacuolation by colchicin, which was considered to be one of the spindle poisons. These findings suggest that components of the Golgi region has intimate relation to the mitotic apparatus.

According to Ris (44), the spindle in the living state is gelatinous, semi-solid body and is never penetrated by cytoplasmic granules. Recently some authors (32, 42), on the basis of the phase contrast and electron microscopic observations, objected to these conceptions concerning the nature of the spindle. From the experimental results that the spindle can be moved in the cell by the use of centrifugal force (47) or even isolated from the cell (39), it may be infered that the living spindle consists of its groundsubstances (59) and spindle fibers, and both are different in quality from cytaplasma.

On the other hand, cytochemical investigation revealed that the spindle had different constituents from the surrounding cytoplasm. There is little RNA in the spindle region of animal cells (31, 47). Davies (16), who investigated UV photomicrographically the living cultured fibroblasts, demonstrated the lower density of absorbing substances in this region. In the present experiment, the region which coincides with the spindle in location is also relatively non-absorptive. This spindle region is transformed from the area into which the juxtanuclear non-absorbing

* The author is grateful to Dr. H. Yamada, in our depatment, for his help with the electron microscope.

region and non-chromosomal nuclear substances have conglomerated together. It seems difficult, under these findings, to avoid the conclusion that the Golgi region is involved in the spindle formation, although the mechanism of this process is not yet clarified.

As to the origin of spindle materials, Ris (44) and Stich (49, 50) reported that they were stored in the nuclear sap just before the onset of mitosis. From the analytical comparison with protein components in both the spindle and nuclear sap (40), proteins must be synthesized somewhere in order to form the spindle. Recently Stich (52) stated that RNA was necessary to form the spindle fibers in his "3rd protein synthesis system". As to the origin of the intranuclear RNA, first of all, nucleolus may be considered. The nucleolus disappears during mitosis and reappears when mitosis is over. According to Fujii (21), Zn which is contained only in the nucleolus appears in the spindle when the nucleolus has disappeared. This fact suggests that the nucleolus might have something to do with spindle formation. The results of the present experiment also suggest that the UV absorbing materials contained in the less absorbing substances of nucleolus may contributes in some way to the formation of the spindle.

UV absorbing substance in the interzonal region: The origin of the UV absorbing substance which appears in the interzonal region during anaphase cannot be determined by the present experiment alone. However, the absorption both at 265mμ and at 280 mμ give an assumption that this substance is different in quality or quantity from that of chromosomes. This substance reveals the nature of RNA on the staining reaction. Davies (16) demonstrated the high sensitivity of UV irradiation at the interzonal absorbing materials of living chick fibroblasts. Jacobson and Webb (31) reported that this substance was RNA originating from the nucleolus, and that it was transfered from the chromosomes into the cytoplasm during anaphase. Ris (44) described that this RNA might be related to the appearrance of interzonal fibers. As mitosis proceeds, the density of the interzonal absorbing materials decreases until it becomes the same as that of the other part of cytoplasm, and then the cytoplasm divides to complete mitosis. If the interzonal region keeps on decreasing in density and finally becomes non-absorptive, the cytoplasm can never divide. From this fact it may be concluded that the absorbing substance of the interzonal region also is one of the factors that determine cytokinesis.

Nucleolus: Altman (3), who investigated the pancreatic cells of mice with the phase contrast microscope, described that the nucleolus was an accumulation of "Nucleolarsubstanz" in the chromosomes, and that it was structureless and had "Chromosomal Randschale" around it like a fringe. Casperson (10) also defined that the nucleolus was a "Depót" of proteins and RNA, and was structureless. On the other hand, since the middle of the last century there has been a statement

strongly contradictory to the notion that the nucleolus is structureless (25). The statement insists that the nucleolus consists of two elements. Since Estable and Sotelo (19) have termed the nucleolonema as a nucleolar inclusion, several investigators observed similar structure to it with the phase contrast microscope (56) and also in electron micrographs of nucleoli of various cells (6, 28, 33). In some instances, nucleoli have no filamentaous structure, but consist of two regions each different from the other (2, 12, 41, 48, 51). While Estable and Sotelo (20) defined that the nucleolonema was Feulgen-negative and pyronine-positive, Lettre and Siebs (35, 36, 37) advocated that it should be named as "Chromosomalteil" of nucleolus, because it was Feulgen-positive and methylgreen-positive in the cultured fibroblasts and tumor cells. According to Lettre and Siebs, "Nucleolarsubstanz" is attached to the swollen "Chromosomalteil", whose shape varies according to the given condition. For instance, when the latter is covered thoroughly by the former, it is difficult to find the latter. The facts mentioned above indicate that the Feulgen-positive element is always presented in the nucleolus, even when nucleolonema does not assume an obvious filamentous structure in it. Ludford (38) also pointed out the Feulgen-positive element in the nucleoli of malignant tumor cells and termed it "Nucleolar chromatin", which varys in size and form.

The findings of the nucleolus in the living Yoshida sarcoma cells may only be due to the difference in concentration of UV absorbing materials. However, the observation on its behavior during mitosis lead author to conclusion that one element of nucleolus is different from the other both in chemical constituents and in function. With May-Giemsa stain (Jacobson and Webb's method using smeared freezing substitution method for fixation), the blue homogenous material in nucleolus is distinguishable from reddish-violet structural element. Moreover, the former is Feulgen-negative and stained reddish with pyronine-methylgreen stain, while the latter is Feulgen-positive and stained green.

From the facts mentioned above, the heavily absorbtive substances of nucleolus correspond to "Chromosomalteil", "Nucleolar chromatin", "Nucleolus-associated chromatin" (10) or "Randschale", and they seem to be a part of resting chromsomes, while the less absorbtive substances correspond to "Nucleolus" (Casperson and Altmann), "Plasmosomin" (Ludford) or "Pars amorpha" (Estable and Sotelo), and the latter seem to be the nucleolar substances in a narrow sence. According to a given functional state of the cell, the less absorbtive substances may change in density or quantity. Therefore, in a very extreme case nucleoli may be seen as homogenous and strong absorptive bodies.

Conclusion

The living Yoshida sarcoma cells were investigated with the UV microscope.

The cytoplasm of the resting cell exhibits heavy absorption except at the juxtanuclear region, which is non-absorbing. This area corresponds to the Golgi region, and during mitosis it transforms into the spindle region together with the non-chromosomal substance of nucleus. At the early stage of anaphase the interzonal region absorbs strongly. The absorbing materials of this region, which lose their density as anaphse proceeds, are considered as one of the factors that determine cytokinesis. The nucleolus of this tumor cell consists of two elements; one is dot- or rod-like or irregularly shaped globules showing heavy absorption, and the other is less absorptive and homogeneous, occupying the interspaces of the former. The former seems to be a part of resting chromosomes, while the latter seems to be the nucleolar substance in a narrow sense.

Acknowledgments

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Explanation of Plates XIV—XVI

Fig. 1: Showing the resting cell photographed at 265 mμ (1a). Note the nucleolar absorption and the juxtanuclear non-absorbing region at the niche of the nucleus. Two nucleolar elements are more conspicuous but the juxtanuclear non-absorbing region is less so at 280 mμ (1b). In phase contrast microphotograph (1c) the area corresponding to the juxtanuclear non-absorbing region is not a clear region.

Fig. 2: Resting cells. 265 mμ (2a), 280 mμ (2b).

Fig. 3: The juxtanuclear non-absorbing region is on the side nearest to the center of the center of the cell. 265 mμ.

Fig. 4: Nucleus is irregularly shaped. The juxtanuclear non-absorbing region is located at the upper side. 265 mμ.

Fig. 5: Binucleated cell. The juxtanuclear non-absorbing region is between the nuclei. 265 mμ.

Fig. 6: Multinucleated cell. The juxtanuclear non-absorbing region is amoung the nuclei. 265 mμ.

Fig. 7: Showing the cytoplasmic vesicles. 265 mμ.

Fig. 8: Neutral red positive granules are in the juxtanuclear non-absorbing region. 265 mμ (8a), 546 mμ (8b).

Fig. 9: Showing the two elements of the nucleolus. 265 mμ (9a), 280 mμ (9b).

Fig. 10 to Fig. 13: Prophase, 265 mμ.

Fig. 14: Prophase, (14a) 0 min. (14b) 24 min. (14c) 38 min. 265 mμ.

Fig. 15: Metaphase. 265 mμ.

Fig. 16: Metaphse. 265 mμ. Note the cytoplasmic absorption.

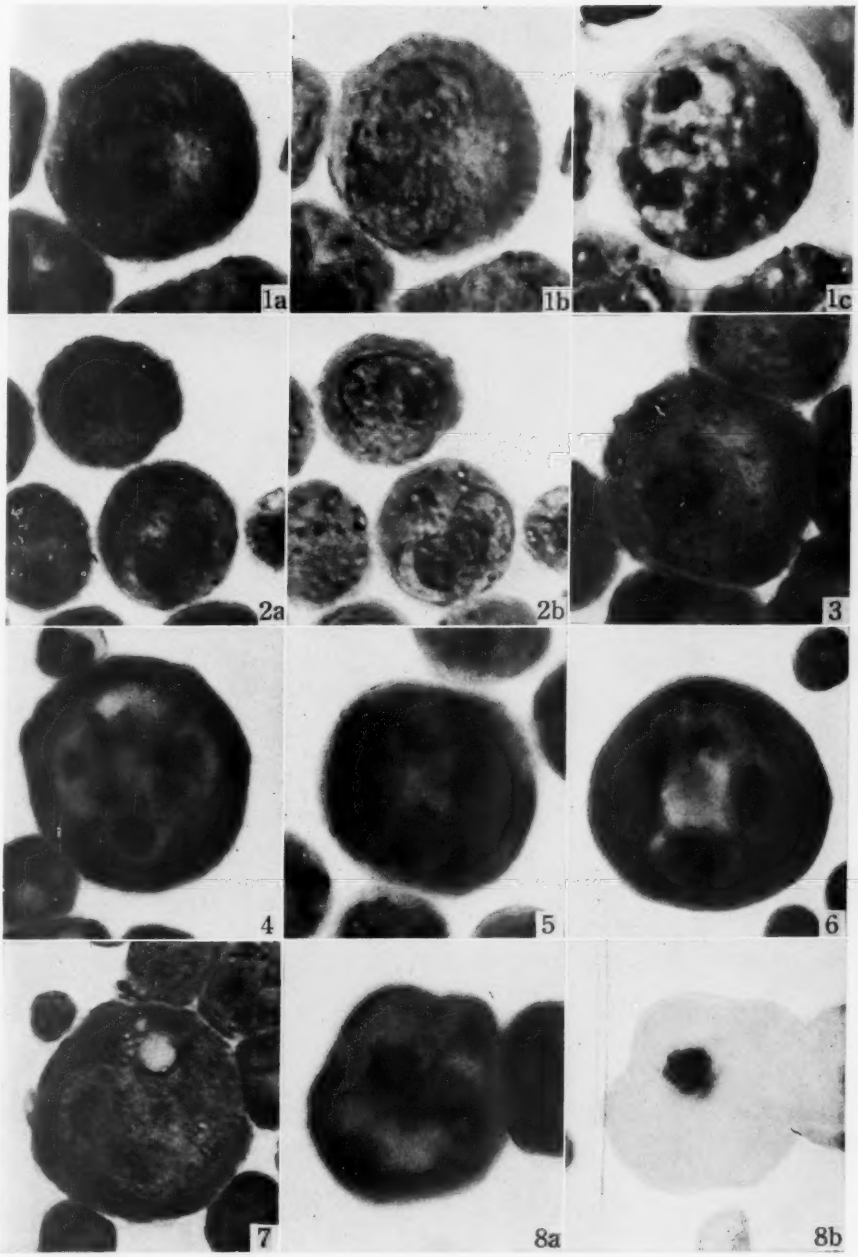
Figs. 17, 18: Metaphase. 265 mμ.

Fig. 19: Anaphase, showing the absorption in the interzonal region. 265 mμ (19a), 280 mμ (19b).

Fig. 20: Anaphase, interzonal absorption (20a) is lost after 4 min. 265 mμ.

Fig. 21: Telophase. 265 mμ. (21a) 0 min. (21b) 23 min. (21c) 113 min.

Fig. 22: Binucleated cell formation. 265 mμ. (22a) 0 min. (22b) 36 min. (22c) 80 min.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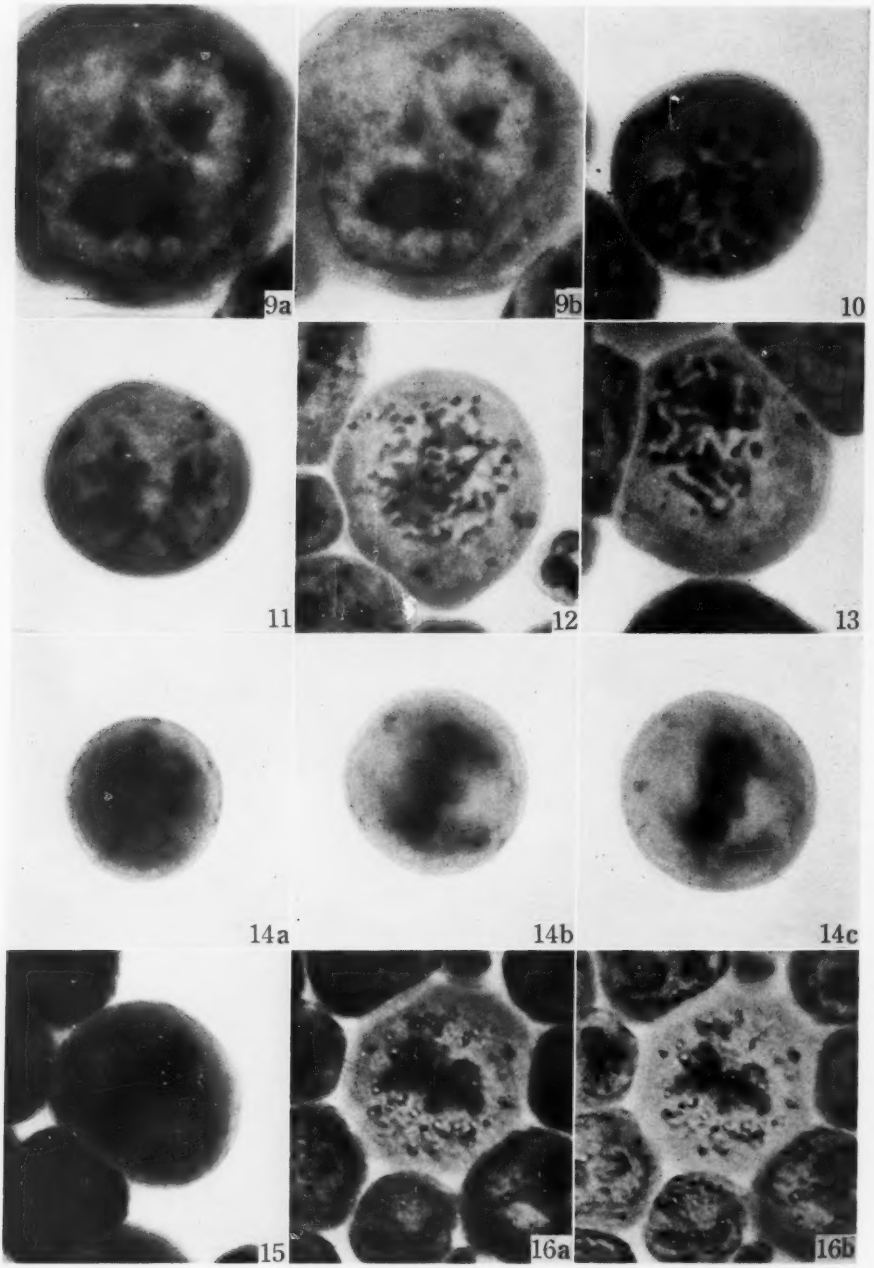
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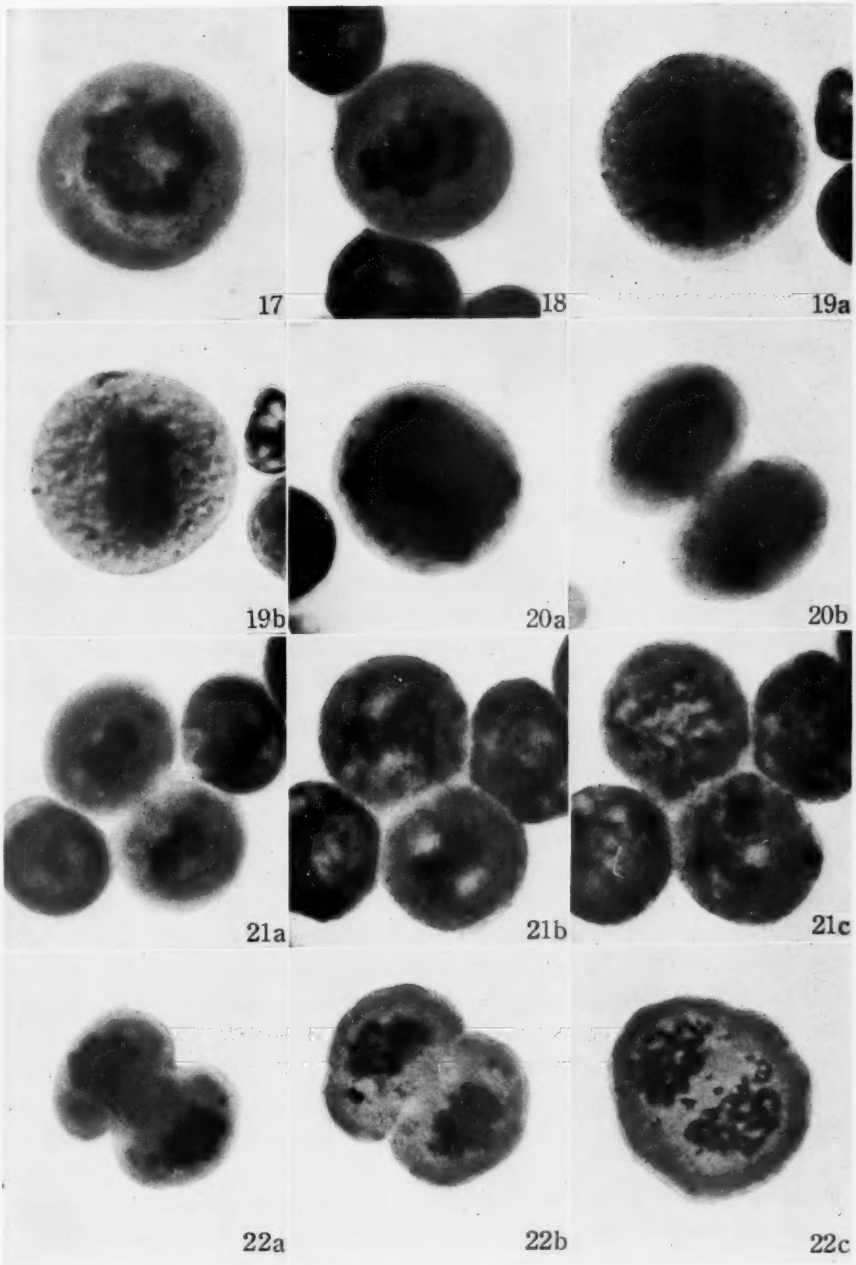
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[GANN, 51, 179~185; June, 1960]

COMPARATIVE STUDIES ON HISTOLOGICAL FEATURES OF THE ORIGINAL STRAIN AND RESISTANT SUBLINE TO NITROGEN MUSTARD N-OXIDE IN THE ASCITES HEPATOMA

(Plates XVII and XVIII)

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Development of resistance in experimental tumors to various anti-tumor drugs has already been studied by many workers. However, little information is available on difference in morphological features between the original strain and resistant subline. Law[1] has reported that no difference could be found in histological features between the original sensitive strain and resistant subline to folic acid antagonist in mouse leukemia L 1210. The present author[2-6] also reported that there was no difference in histological features of the original strain of the Yoshida ascites sarcoma and its resistant subline to methyl-bis-(β-chlorethyl)-amine N-oxide (Nitrogen mustard N-oxide) (MBAO). However, these results were obtained with nonepithelial tumor. In the present study the author attempted to establish a resistant subline to MBAO, using the ascites hepatoma, which is of epithelial nature, and to compare its histological features with those of the original strain.

Procedure and Results

The ascites hepatoma established by Yoshida[7] was used in this study. As reported by Yoshida and Kurata[8], this tumor is not a free cell tumor in the strict sense of the word. The tumor cells, being epithelial in nature, make cell associations, or "islands", suspended in the ascitic fluid, although individually isolated cells are also present. The number of these isolated cells differs, depending upon the hepatoma strain. Tumor "islands" continue to increase in number, with widespread invasion into the peritoneal tissues, resulting in death of the host animals in about two weeks after inoculation. A resistant subline of each of two strains of the ascites hepatoma, AH 13 and AH 7974, to MBAO was established according to the same methods as described in previous paper.[2] Rats of mixed breed and weighing about 100 g were used, which were highly susceptible to this tumor. They were given intraperitoneal inoculation of about 0.1 ml of undiluted tumor ascites in a state of "nearly pure culture".

Development of resistance to MBAO in AH 13.——In the 1st transfer generation, rats received daily subcutaneous injections of MBAO, beginning on the 4th day after inoculation, until they died of tumor. Since the 2nd transfer generation,

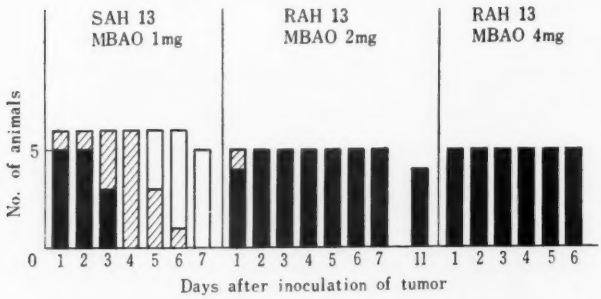

CHART 1. Comparison of susceptibility to MBAO between original strain AH 13 (SAH 13) and resistant subline (RAH 13). All rats received subcutaneous injections of MBAO every other day from just after intraperitoneal inoculation with 0.05 ml of tumor ascites. MBAO 1 mg, 2 mg, and 4 mg represent a single injection dosage of MBAO respectively. Black bars, "nearly pure culture" of tumor cells; cross-hatched bars, only few tumor cells; open bars, no tumor cells.

injections were commenced just after inoculation and continued in the same way as in the 1st transfer generation. Transmission of tumor to the next generation was made using about 0.1 ml of tumor ascites withdrawn just before the death of animals. Daily dosage of MBAO was as follows: 50 μg/100 g body weight in the 1st generation, 250 μg in the 2nd generation, 500 μg in the 3rd generation, 1 mg from the 4th to 7th generation. A single dosage of MBAO was dissolved in 0.5 ml of saline. In the 7th transfer generation, about 60 days after the beginning of injection in the 1st generation, tumor cells showed complete resistance to the treatment with daily dosage of 1 mg of MBAO. Furthermore, in the 10th transfer generation they showed resistance to the same treatment with daily dosage of 3 mg. Comparison of susceptibility to MBAO between the resistant subline and original strain is shown in Chart 1. In animals which received the inoculation of original strain, only few tumor cells survived on the 4th day after inoculation and on the 7th day tumor cells disappeared completely from the peritoneal fluid in all cases, when a single dosage of 1 mg of MBAO was subcutaneously injected every other day from just after intraperitoneal inoculation with 0.05 ml of tumor ascites. On the other hand, in case of resistant subline, tumor cells remained in a state of "nearly pure culture" in spite of the same injection with single dosage of 2 mg or 4 mg. As described above, this subline showed a resistance to consecutive subcutaneous injections with 1 mg in the 7th transfer generation and to those with 3 mg in the 10th transfer generation. Since the aim of the present experiment was in establishing resistant subline to study the histological features, the author has no sufficient data concerning the mode of

development of resistance. However, the mode of development in AH 13 seems to be somewhat different from that in the Yoshida ascites sarcoma, i.e., development of resistance in AH 13 seems to resemble the streptomycin pattern of resistance in bacteria,[9,10] while that in the Yoshida ascites sarcoma arises in stepwise fashion resembling the penicillin pattern of resistance[2]. It may be presumable that the mode of development of resistance to a certain anti-tumor drug varies depending upon the tumor strain. Experiments on this problem are now in progress. A more detailed report will be published elsewhere.

Development of resistance to MBAO in AH 7974. In the 1st transfer generation, daily subcutaneous injections with 1 mg of MBAO was commenced from just after inoculation with tumor. From the 2nd generation to 6th, daily dosage of 2 mg was injected, and in the 7th generation daily dosage of 3 mg was administrated in the same manner as in the 1st transfer generation. Finally, since the 8th transfer generation daily dosage of 4 mg was administrated. Complete resistance to consecutive injections with daily dosage of 4 mg from just after inoculation developed in the 11th transfer generation, four months after the experiment was started. However, since animals die of toxic effect of MBAO within a short period, it was impossible to develop the resistance in much higher degree *in vivo*. Strain AH 7974 shows originally a strong resistance to MBAO as confirmed by Yoshida[7]. Then, comparison of susceptibility of the original strain and resistant subline was carried out according to the following methods: 1) a single intraperitoneal injection with 5 mg of MBAO was made just after inoculation with 0.2 ml of undiluted tumor ascites and 2) a single intraperitoneal injection with 6 mg was made just after inoculation

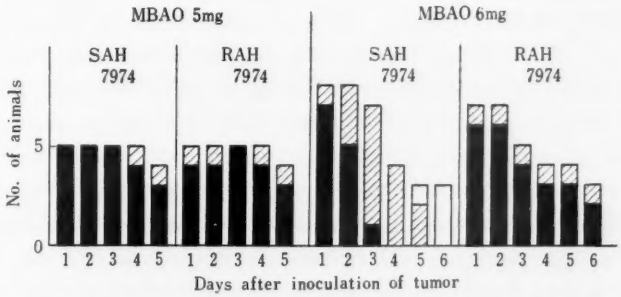

CHART 2. Comparison of susceptibility to MBAO between original strain AH 7974 (SAH 7974) and resistant subline (RAH 7974). All rats received intraperitoneal injection of MBAO just after inoculation of tumor, i.e., animals inoculated with 0.2 ml of tumor ascites received a injection with 5 mg of MBAO and another group of animals inoculated with 0.05 ml of tumor ascites received a injection with 6 mg of MBAO.

with 0.05 ml of undiluted tumor ascites. In the first experiment, tumor ascites of most animals which received the inoculation of the original strain and resistant subline respectively, remained in a state of "nearly pure culture", without any remarkable decrease of tumor cells in number (Chart 2). However, original strain showed much more abundantly signs of degenerative cells than resistant subline. In second experiment, clearer difference was observed in susceptibility between these two lines (Chart 2). Although each resistant subline of AH 13 and AH 7974 has been transfered successively in host animals not given MBAO for more than five months, resistance remained unchanged as observed in the resistant subline of the Yoshida ascites sarcoma[3].

Ascitic findings and histological features of the original strain and resistant subline of AH 13. All observations were carried out using rats without MBAO treatment. Observations on ascitic findings were performed using squash preparations with aceto-dahliaviolet and Giemsa stained smear preparations of the tumor ascites. Histological observations were made using the tumor tissues invaded into the omentum of animals that were left to die of the tumor. After fixation in 10 per cent formalin, sections were stained mainly in hematoxylin and eosin. Observations on glycogen were made according to periodic acid schiff staining. As to the ascitic findings of AH 13, Kurata[8] reported that 99.8 per cent of total tumor cell units was single free tumor cells and remaining 0.2 per cent was "islands" composed of 2 to 10 tumor cells. However, the present author has not encountered "islands" in AH 13. All of tumor cells in the resistant subline were also single free tumor cells. Significant difference in cytological feature of the original strain and resistant subline was not observed. Also glycogen contents of tumor cells were almost the same in these two lines. Moreover, a significant difference in histological features also was not observed between the original strain and resistant subline (Plate, Figs. 1 and 2). Both lines showed generally a sarcomatous appearance without any peculiar arrangement of tumor cells which are of round shape, some with anastomosing cytoplasmic processes and others with distinct cell boundaries. Cell aggregates or solid alveolar arrangement was not detected anywhere.

Ascitic findings and histological features of the original strain and resistant subline of AH 7974. Generally, the frequency of single isolated tumor cells and size of "islands" in ascitic fluid are not always constant depending upon the conditions of host animals. Accordingly, it is difficult to compare the resistant subline of AH 7974 with its origin strain, regarding the frequency of single tumor cells and size of "island". However, a relatively clear difference was observed between these two lines (Figs. 3, 4 and Table 1). Frequency of single isolated tumor cells and number of tumor cells which compose "islands" were studied using tumor ascites in a state of "nearly pure culture" after inoculation. As seen in Table 1, single

Table 1. Frequency of single isolated tumor cells and number of tumor cells which compose "islands" in the original strain and resistant subline of AH 7974.

Strain	Total number of single tumor cells and "islands"	Single tumor cells	"Islands" composed of			
			2–10 cells	11–20 cells	21–50 cells	more than 51 cells
Original strain	860	99 11.5%	639 74.3%	83 9.6%	36 4.1%	3 0.3%
Resistant subline	829	82 9.8%	278 33.5%	236 28.4%	179 21.5%	54 6.5%

isolated tumor cells and "islands" composed of small number of tumor cells, 2 to 10 tumor cells, were fewer in resistant subline than in the original strain. On the contrary, "islands" composed of more than 11 tumor cells were observed much more frequently in resistant subline than in the original strain. The histological structure of AH 7974 invaded into the omentum is, as a rule, composed of the following two structures, i.e,, 1) diffuse arrangement of pleomorphic tumor cells, polyhedral, round or elongated cells, without any peculiar structure (Fig. 5) and 2) solid alveolar arrangement or cell aggregates, groups of tumor cells arranged in cords and islands (Figs. 6 and 7). The latter structure showed frequently that of adenocarcinoma or papillary cystoadenocarcinoma owing to the disintegration of tumor cells in the central portion (Fig. 8). There was no fundamental difference in these histological appearance between original strain and resistant subline. In original strain, however, the features of diffuse arrangement of tumor cells were predominant comparing with those of alveolar arrangement, cell aggregates, or adenocarcinoma (Fig. 5). Even in parts showing a certain architecture, it was mainly in a trabecular fashion (Fig. 6). On the contrary, in the resistant subline, features of solid alveolar arrangement, cell aggregates, and glandular structure were much more predominantly observed than diffuse arrangement of tumor cells (Figs. 7 and 8).

Discussion

Law[1] reported that there was no difference in histological features between mouse leukemia L 1210 and its resistant subline to folic acid antagonists. The present author also studied the histological features of the Yoshida ascites sarcoma and its resistant subline to MBAO, but no difference could be detected between these two lines[6]. Both L 1210 and the Yoshida ascites sarcoma are nonepithelial tumor. In the present study, AH 13 and AH 7974 which are epithelial tumors were used as material. Resistant subline of AH 13 which is composed of only single isolated tumor cells did not show any significant difference in either ascitic or histological feature as compared with the original strain. However, histological features of

resistant subline of AH 7974 which is composed of "islands" and single isolated tumor cells, showed groups of tumor cells arranged in islands and glandular structures much more predominantly than in the original strain. Furthermore, it was confirmed in ascitic findings that "islands" in resistant subline were composed of more tumor cells than those in the original strain. The author reported previously that motility and mutual adhesiveness of tumor cells are imortant factors in forming histological structures of the tumor and that active motility and decrease in mutual adhesiveness result in diffuse arrangement of tumor cells without showing any peculiar arrangement, while increase in adhesiveness and inactive motility result in forming the alveolar arrangement of tumor cells[11]. From these points of view, it may be conceivable that there is a difference in adhesiveness and motility of tumor cells between original strain of AH 7974 and its resistant subline. Studies on these points are now in progress. Furthermore, that there was no significant difference in histological features of original AH 13 and its resistant subline may due to the fact that AH 13 is composed of only single isolated tumor cells.

Summary

Using two strains of the ascites hepatoma, AH 13 and AH 7974, which are of epithelial nature, resistant sublines to methyl-bis-(β-chlorethyl)-amine N-oxide were established by passing tumor cells through animals treated with the agent. There was no significant difference in ascitic findings and histological features of tumor tissues between the original strain and resistant subline of AH 13 which is composed of single free tumor cells. However, in AH 7974 in which tumor ascites is composed of cell aggregates and single isolated tumor cells, a significant difference was observed in both ascitic findings and histological features between the original strain and resistant subline. These differences were attributed to changes in motility and mutual adhesiveness of tumor cells.

This investigation was supported in part by a grant from Japan Cancer Society.

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Explanation of Plates XVII and XVIII

Fig. 1. Tumor tissues of original strain of AH 13, 7 days after inoculation. ×550.

Fig. 2. Resistant subline of AH 13, 11 days after inoculation. ×550. No significant difference was observed in histological features between the original strain (Fig. 1) and resistant subline. Both two lines showed generally a sarcomotous appearance without showing any peculiar arrangement of tumor cells.

Fig. 3. Ascitic findings of the original strain of AH 7974, 8 days after inoculation. Squash preparation with aceto-dahliaviolet. ×140. Besides single isolated tumor cells, many "islands" composed of small number of tumor cells can be seen.

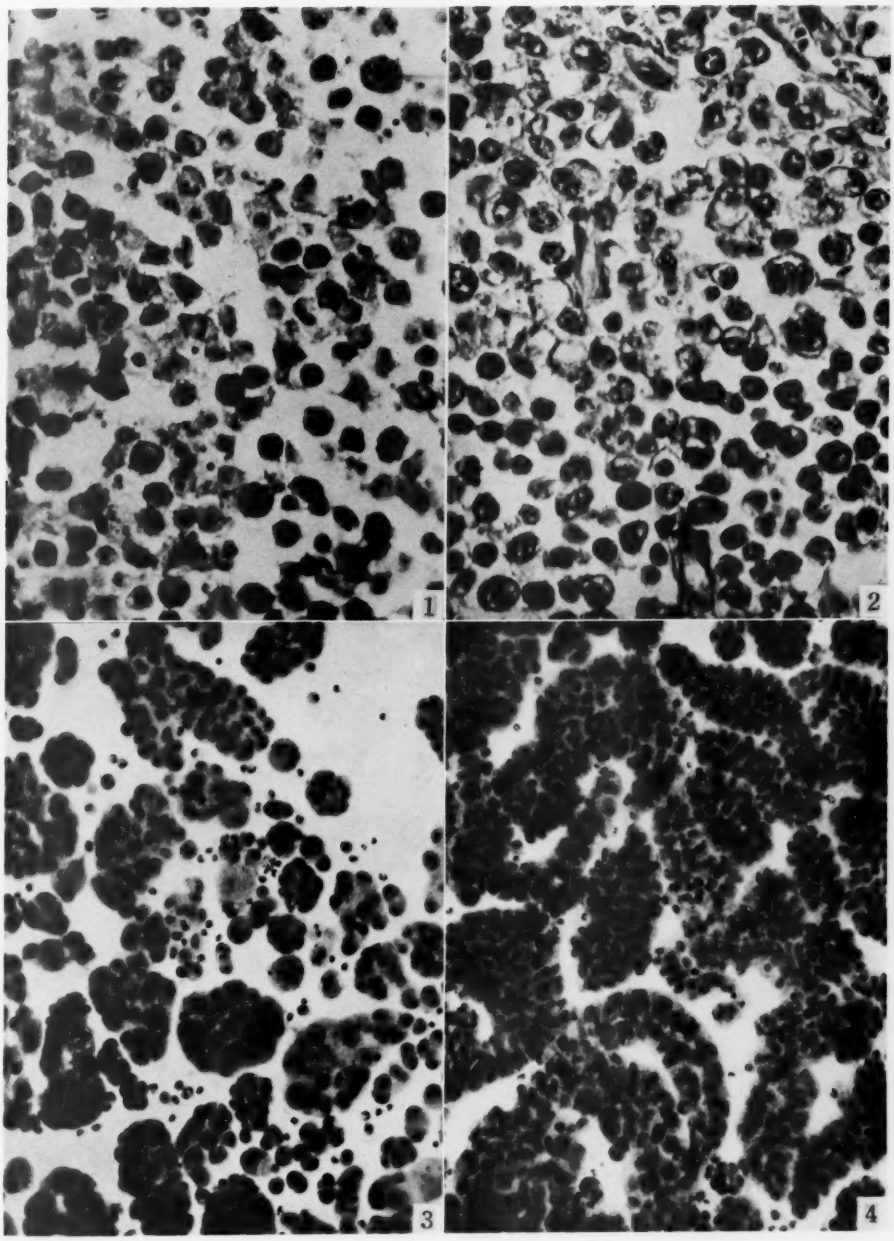
Fig. 4. Ascitic findings of the resistant subline of AH 7974, 8 days after inoculation. Squash preparation with aceto-dahliaviolet. ×140. Most "islands" are composed of more tumor cells than those in the original strain (cf. Fig. 3).

Fig. 5. Tumor tissues of the original strain of AH 7974, 9 days after inoculation. Diffuse arrangement of tumor cells without any peculiar structure. ×140.

Fig. 6. Original strain of AH 7974, 9 days after inoculation. Both diffuse arrangement of polyhedral, round or elongated tumor cells and groups of tumor cells arranged in trabecular fashion are seen. ×275.

Fig. 7. Resistant subline of AH 7974, 9 days after inoculation. Solid alveolar arrangement or cell aggregates like as "islands" in ascitic fluid is observed predominantly. ×140.

Fig. 8. Resistant subline of AH 7974, 7 days after inoculation. Groups of tumor cells arranged in islands and glandular structures of tumor are observed. ×140.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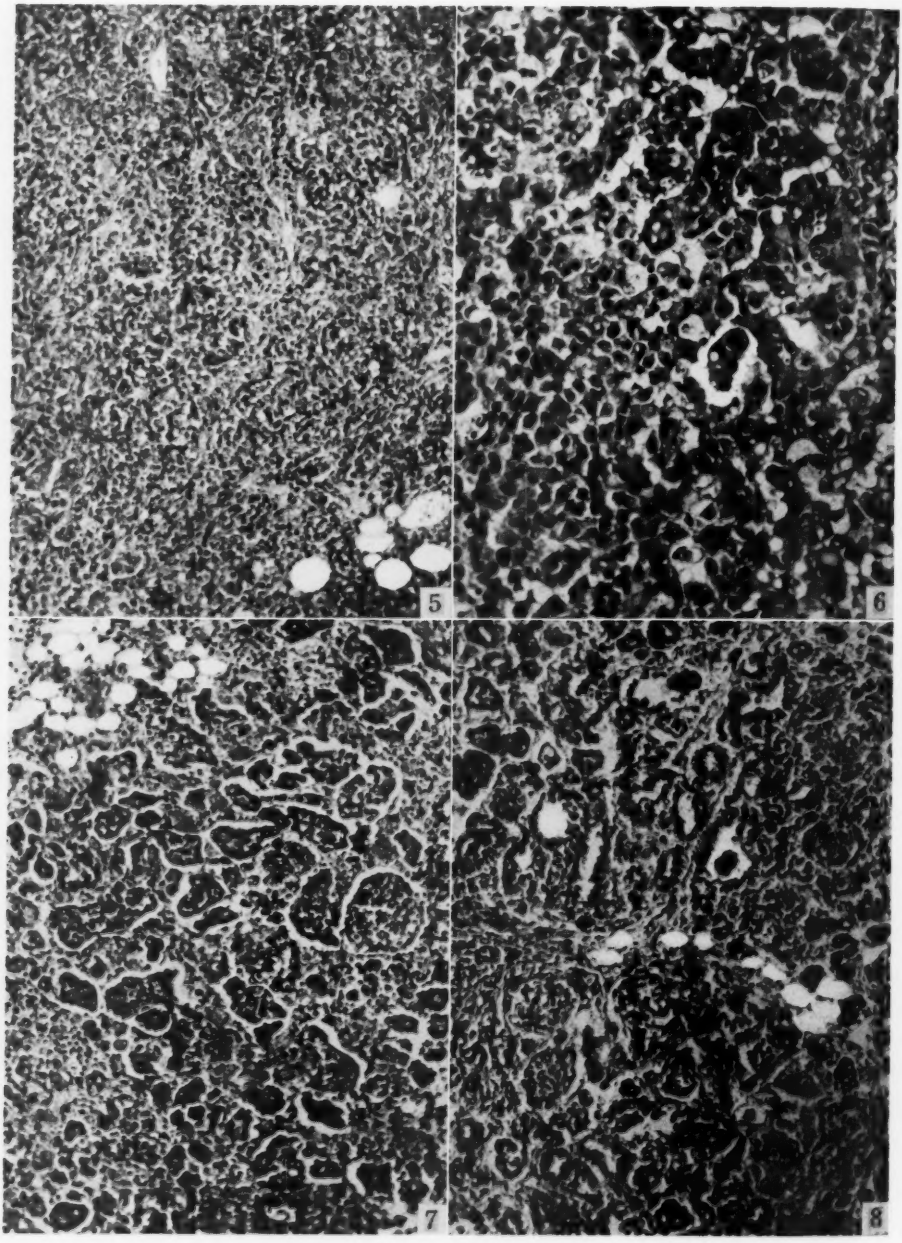
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[GANN, 51, 187～199; June, 1960]

STUDIES ON THE MODE OF ACTION OF RC4, A NEW ANTI-CANCER AGENT CONTAINING ETHYLENEIMINO RING I. EFFECTS OF RC4 AND ALLIED AGENTS ON GLYCOLYSIS, RESPIRATION, HEXOKINASE AND PHOSPHATASE OF EHRLICH ASCITES TUMOR CELLS AND ON CHOLINE-ESTERASE OF HUMAN BLOOD

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RC4 synthesized in our laboratory is an alkylating agent having ethyleneimino rings.[1] At first Uchida et al.[2] recognized the anticancer effect of this agent upon Ehrlich cancinoma of both ascitic and subcutaneous tumor types and upon transplanted mammary cancer of C_3H- and A-mice, and later its effectiveness against various animal cancers was confirmed at Sloan Kettering Institute for Cancer Research in U.S.A.[3] On the other hand, Katsura et al.[4] obtained a complete cure of Yoshida sarcoma by subcutaneous injection of RC4 in combined with 1-thia-3-azazulene-2-one. Subsequently many other workers re-examined and confirmed the anti-cancer effect of RC4 upon animal cancers. Its therapeutic effectiveness is now under investigation with a promising result.

Generally speaking, an alkylating agent reacts as an electrophilic reagent under mild conditions in aqueous solution, that is, under physiological conditions;[5] hence it is presumed that the alkylating agent reacts with functionally important nucleophilic centers in biological systems and inhibits the physiological actions. However, despite the fact that SH group is a typical nucleophilic reagent, it was only phosphokinase, among the so-called SH-enzymes, that proved sensitive to N-mustards;[6] therefore the action of alkylating agent seems to be rather selective. For these reasons, the action of alkyating agent on various enzymes or enzyme systems has been examined up to now, yet no clear-cut conclusion has been attained.

While many scholars regard the inhibitory activity of alkylating agent against

The following abbreviations are used in the present paper. RC4=0, 0′-*p*-phenylene-N, N′,N″, N‴-tetraethylenetetraimido-diphosphate; *o*-RC4=0, 0′-*o*-phenylene-N-ethyleneimido-phosphate; *m*-RC4=0, 0′-m-phenylene-N, N′, N″, N‴tetraethylenetetraimido-diphosphate; TEPA=N, N′, N″-triethylenephosphorade; NMO=nitrogenmustard-N-oxide; TEM=2, 4, 6-triethyleneimino-1, 3, 5-triazine; E39=2, 5-bisethyleneimino-3, 6-propioxybenzoquinone (1, 4); ATP=adenosinetriphosphate; ATP-ase=adenosine-triphosphatase; Ace-Ch=Acetylcholine chloride; ChE=cholinesterase; TCA=trichloracetic acid: PNPP=sodium-*p*-nitrophenylphosphate.

protein and nucleic acid metabolism as the essential factor in its anti-cancer action, not a few students, such as Roitt[7] or Holzer et al.[8-11] suggest that the anti-cancer activity of the ethyleneimines may be attributed to their lowering action on DPN lebel and thus to cause the inhibition of tumor cells.

Whichever may be the truth, bio-synthesis of nucleic acid or protein, which requires energy, may be retarded from the inhibition of glycolysis or respiration. For this reason, it seems reasonable to start the study on the mode of action of a new agent such as RC4 with examination of its effect on the energy-generating systems.

The present report dealts with glycolysis and respiration (endogenous) of Ehrlich ascites tumor cells as the energy-generating systems, and ChE and phosphatase which are confirmed to be remarkably affected by some alkylating agents.[12,13] The effects of RC4 on these enzymes or enzyme systems were then examined in comparison with those of *o*-RC4*, TEPA and NMO.

NMO and *o*-RC4 displayed remarkable inhibition of glycolysis and stimulation for alkaline-phosphatase, whereas RC4 and TEPA scarcely showed any such action. However, when the aqueous solutions of the latter two were preserved at 2°C and their action upon the above-mentioned enzymes wes examined at certain intervals, it was observed that the activities of RC4 and TEPA gradually increased, eventually to the same degree as NMO. We will report these findings and discuss their significance.

Materials and Methods

Chemicals: RC4, *o*-RC4 and TEPA were prepared in this laboratory by Dr. Genshun Sunagawa. RC4 and TEPA used were pure crystals. *o*-RC4 was amorphous powder. Though highly refined, it might contain a more or less degenerated portion as it was somewhat unstable. Each of the above agents was stored in vacuo at temperature below 10°C. The sample of NMO used was "Nitromin" crystals for injection (Yoshitomi Pharmaceutical Company), which was neutralized by diluted NaOH aqueous solution to prepare the 2.5% solution when used. Other agents were used as 10% aqueous solution. Each solution was preserved at 2°C. Since the preserved solution of RC4 tends to be more or less acid, it was neutralized immediately before test. Other solutions were used as they were or after being properly diluted.

ATP (disodium salt) was a preparation from Zellstoffabrik.

Tumor cells: The Ehrlich asites tumor cells were transplanted into dd-mice (Hamamatsu Farms), and harvested on 7 to 9 days after inoculation.

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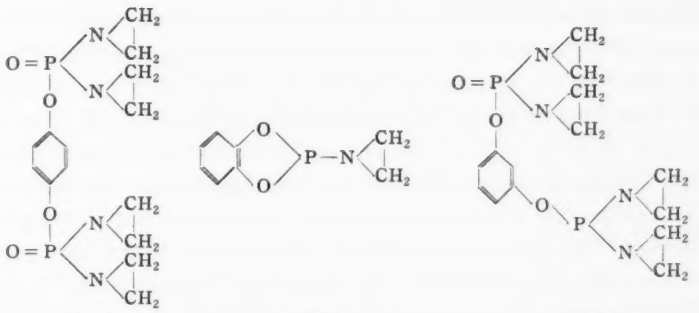
* Although the anticancer activity was confirmed against animal cancer,[14] clinical study of *o*-RC4 is not yet tried on account of its low stability.



RC4 o-RC4 m-RC4

Measurement of Endogenous Respiration and Glycolysis: Kun and his collaborators' method[15] was followed. After blood-corpuscles were removed from the ascites tumor cells by washing 3 or 4 times with ice cold Krebs-Ringer solution, for measurement of respiration and aerobic glycolysis the cell suspension containing 5×10^7 cells/ml and for that of anaerobic glycolysis the suspension containing 2.5×10^7 cells/ml were used, prepared by dilution with a proper buffer solution. Determination of respiration and anaerobic glycolysis was conducted by the Warburg manometric method. In the respiration experiment, 1.7 ml of Krebs-Ringer phosphate (pH 7.4), 0.3 ml of water or additions and 1.0 ml of the cell suspension were put into the main flask, and 0.2 ml of 20% KOH and filter paper roll into the center well. The gas phase was air. The respiratory quotients was determined by direct method,[16] CO_2 retention at the beginning and end of the run being measured by tipping 0.20 ml of 6N-H_2SO_4 from the side arm into the main flask. Anaerobic glycolysis was determined in Krebs-Ringer bicarbonate solution with 95% N_2-5% CO_2 at the gas phase. Each flask contained 1.7 ml of Krebs-Ringer bicarbonate, 0.3 ml of water or additions, and 1.0 ml of the cell suspension in the main flask, and in the side arm 0.2 ml of 1.5% glucose of fructoese solution (16.6 μmoles*), which was tipped into the main flask after temperature equilibration.

In some experiments, manometric determinations were checked by chemical determinations[17] of the lactic acid, present in the flasks at the beginning and end of the experiment. For mersurement of aerobic glycolysis, a Monod-type shaking culture equipment was used; 3.1 ml of the Krebs-Ringer phosphate, 0.5 ml of water or additions, and 1.0 ml of the cell suspension were put into the L-shape tube and, after temperature equilibration, 0.4 ml of 1.5% glucose was tipped. At the beginning and end of the reaction, 2.0 ml of 20% TCA was added to the reaction mixture, and the quantity of lactic acid formed was analyzed.

* M, mM or μM are used as units of concentration, and mole, mmole, or μmole as those of quantity, for the sake of distinction.

Determination of Hexokinase Activity: By the method of Kun et al.[15] acetone-dried powder of the tumor cells was prepared and, its aqueous extract being used as the enzyme solution, hexokinase activiy was determined by the manometric method.[18] Each main flask contained 0.5 ml of the enzyme solution, 1.0 ml of 0.06M $NaHCO_3$, 0.2 ml of 0.1 M $MgCl_2$, 0.2 ml of 4.5% glucose or fructose solution and 0.3 ml of water or the aqueous solution of RC4, and the final volume was made 2.7 ml by addition of water. Into the side arm were put 0.2 ml of 0.075M-ATP and 0.1 ml of 0.04 M-$NaHCO_3$. The flasks were gassed with 95% N_2–5% CO_2 and, after preliminary shaking, the content of the side arm was tipped into the main flask to start the reaction, and the measurement was conducted. NaF was not added. Blanks containing all the reagents except sugar were run, to correct for the CO_2 evolution due to the acidity or alkalinity of the ATP on tipping and also the ATP-ase activity of the preparation. The CO_2 outputs recorded are corrected for such blank determination.

Determination of Phosphatase Activity: Omori's method[19] was adopted. After washing the tumor cells with 0.9% NaCl the suspension was diluted, in case of determination of acid-phosphatase activity to 3×10^6 cells/ml with citric acid buffer (Sörensen; pH 5.0), or in case of alkaline-phosphatase activity to 2.5×10^7 cells/ml with veronal buffer (Michaelis; pH 9.3); 2.0 ml of the cell suspension and 0.5 ml of water or additions were put into an L-shaped tube and, after temperature equilibration, 2.5 ml of 10^{-3}M PNPP was added. At the end of the reaction, 5 ml of 10% TCA was added to stop the reaction. To 5 ml of the supernatant fluid obtained by centrifugal separation of the deprotenized reaction mixture was added 5 ml of saturated Na_2CO_3 to develop color, and *p*-nitrophenol formed was estimated by measuring the absorption at 430 mμ.

Determination of Human Erythrocytes and Serum ChE Activities: Ammon's manometic method[20] was adopted with a slight modification. Serum was centrifugally separated from 5 ml of defibrinated blood and, after washing erythrocytes three times with Krebs-Ringer solution, the Ringer solution was added to make the total volume 25 ml. The serum was diluted by addition of the equal quantity of Krebs-Ringer solution. Each main flask contained 3.8 ml of Krebs-Ringer solution, 0.3 ml of 4% $NaHCO_3$, 0.5 ml of water or additions, and as a substrate 0.2 ml of 1.25% Ace-Ch in case of erythrocytes, or 0.2 ml of 12.5% Ace-Ch in case of serum, and into the side arm 0.2 ml of diluted solution of erythrocytes or serum. The gas phase was 95% N_2–5% CO_2 and, after a temperature equilibration, the content of the side arm was tipped into main flask, and the CO_2 evolution was measured. CO_2 output of the flask which contained all the reagents but no enzyme preparation was considered to be due to auto-hydrolysis of Ace-Ch, and accordingly was corrected for each determination.

All measurements of respiration, respiratory quotients and glycolysis were carried out at 37.5°C and, after 10-minutes temperature equilibration, the reaction was allowed to proceed for 60-minutes. In manometry, after putting tumor cells in a flask, approximately 30-minutes were required for the reaction to start, and reading was taken at 10-minute intervals.

For colorimetric estimation of lactic acid and *p*-nitrophenol, the Hitachi EPU II spectrophotometer was used.

Definitions of 'Degree of Inhibition' and 'Increasing Degree of Inhibition': 'The degree of inhibition (I)' is calculated by conventional method from the enzymatic activities in the presence and absence of test agent. Plotting I against the logarithmic concentration of the agent, the inhibition curve is drawn. The concentration of the agent corresponding to the 50% inhibition on the curve is indicated as 50% inhibitory concentration (I_{50}). From the 50% inhibitory concentrations at the time of dissolution and after preservation, which are indicated as I^{i}_{50} and I^{o}_{50}, respectively, 'the increasing degree of inhibition' is designated as I^{i}_{50}/I^{o}_{50}.

Results

Effect on Anaerobic Glycolysis: When measured under the above-mentioned conditions, $Q^{N_2}_{CO_2}$ ($\mu l CO_2$/mg dry weight cells/hour) was 36 on an average with glucose as the substrate and 22 with fructose as the substrate. The relative

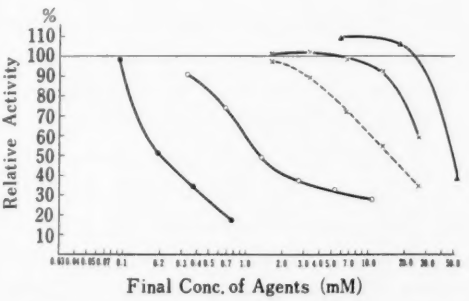


Fig. 1. Relative Activity of anerobic glycolysis of Ehrlich ascites tumor cells incubated with various concentrations of fresh solution of RC4 or related agents. ×——×, RC4 (substrate: glucose); ×···×, RC4 (subatrate: fructose); ○——○, o-RC4; ▲——▲, TEPA; ●——●, NMO; In each case of last three agents, only glucose was used as substrate.

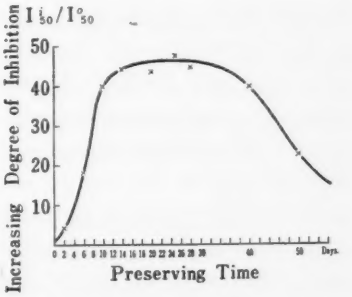


Fig. 2. Effect of preserving of RC4 aqeous solution at 2°C, on its inhibitory activity against anerobic glycolysis of Ehrlich ascites tumor cells. Concentration of the solution at the time of preservation was 10%. Increasing degree of Inhibition was presented as I^{i}_{50}/I^{o}_{50}, where I^{o}_{50} and I^{i}_{50} showed 50% inhibitory dose of observed and initial time of preservation, respectively.

activities of the tumor cells incubated with the agent are shown in Fig. 1. In case of using glucose as the substrate, the relative activity fell to approximately 50% by addition of 2×10^{-4}M of NMO or 1.3×10^{-3}M of *o*-RC4. On the other hand, in case of RC4 or TEPA, the relative activity reached only 50% even at 3 to 5×10^{-2}M or more; the inhibitory activities of the latter two are significantly lower than those of the former two. The use of fructose as the substrate gives a slightly higher inhibitory effect of RC4 than that of glucose does; yet in this case 1.5×10^{-2}M of RC4, that is considerably high concentration is required for 50% inhibition.

Moreover, it was found by chance that when the glycolysis-inhibiting activity of RC4 aqueous solution which had been preserved in a refrigerator at 2°C was measured, the activity was significantly intensified. Then, 10% aqueous solution of RC4 was preserved at 2°C, and the variation in its inhibitory activity against anaerobic glycolysis was examined at intervals. The results are shown in Fig. 2. After 2 weeks of preservation the activity reached its maximum 45 to 50 times as high as that immediately after dissolution. After that, the activity continued to be constant until four weeks later. The solution preserved for 14 to 20 days, which showed the highest activity, will hereinafter be referred to as simply "stock solution", the characteristics of which were compared with those of "fresh solution" immediately after dissolving. As regards other agents, the solution preserved for 14 to 20 days was, for the sake of convenience, called "stock solution", which was compared with the fresh solution. Fig. 3 shows relative activities of anaerobic glycolysis of tumor cells incubated with stock solutions. Not only the inhibitory activity of RC4 but also that of TEPA considerably incrersed, but NMO showed no change and the increase in the activity of *o*-RC4 was nominal. That is to say, it was found that the effect of preservation differed with agents. On ths other hand, the inhibitory activity of RC4 stock solution with fructose substrate proved lower than that with glucose substrate, which was contrary to the result with the fresh solution. Increase of the quantities of the substrates by three times (50 μ moles) scarcely affected the inhibitory activity of the RC4 stock solution.

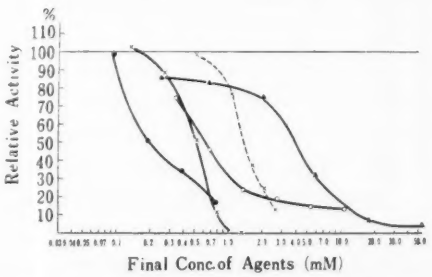


Fig. 3. Relative activity of anaerobic glycolysis of Ehrlich ascites tumor cells incubated with various concentrations of stock solution of RC4 or related agents. ×——×, RC4 (substrate: glucose); ×·····×, RC4 (substrate: fructose); ○——○, o-RC4: ▲——▲, TEPA; ●——●, NMO. In each each case of last three agents, only glucose was used as substrate.

Effect on Aerobic Glycolysis: $Q_{CO_2}^{O_2}$ was about 20 in the absence of agent. In case of fresh solutions, the inhibi-

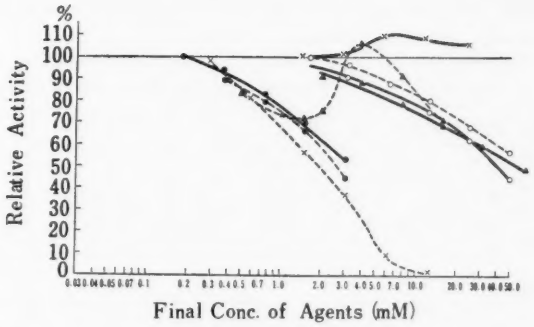


Fig. 4. Relative activity of aerobic glycolysis of Ehrlich ascites tumer cells incubated with various concentrations of RC4 or related agents. ×, RC4; ○, o-RC4 ▲, TEPA; ●, NMO. Solid or dotted line indicates the activity of fresh or stock solution of agent, respectively.

tory activity of NMO was markedly high, and the activities of *o*-RC4 and TEPA were approximately equal. Although RC4 showed no inhibitory activity even at 2.5×10^{-2}M, but displayed a slightly stimulating effect, the inhibitory activity of its stock solution remarkably increased, approximately to the same degree as NMO. The TEPA stock solution displayed also an equal inhibitory activity to NMO in lower concentration, but in high concentration it gave stimulating effect. The activity of *o*-RC4 slightly decreased with preservation.

Effect on Hexokinase: It is well known that N-mustards inhibit hexokinase. Therefore it is probable that hexokinase inhibition is possibly related also to the glycolysis-inhibiting activity of RC4 stock solution. In addition, the variation in the glycolysis-inhibiting activity of RC4 with alternation of substrate, glucose or fructose, might be explained by difference in the inhibitory activities of RC4 against fructokinase and glucokinase.

In order to clarify the above-mentioned points, the experiment indicated in Table 1 was conducted. Since addition of NaF rapidly lowered hexokinase activity with a result of difficulty in accurate measurement, the experiment was made in the absence of NaF, while correcting the result by conducting a control experiment without addition of sugar.

The inhibitory activity of the fresh solution was slightly higher with fructose substrate, which result was consistent with that obtained in glycolysis. However, in case of stock solution, the activity hardly varies with substrates. The inhibitory activity of stock solution is considerably higher than that of fresh solution, but the degree of increase is not so significant as that of increase in the glycofysis-inhibiting activity, and another characteristic is that even the addition of highly-concentrated

Table 1. Effect of RC4 on hexokinase activity of an aqueous extract of an acetone powder of ascites tumor cells.

Experiment No.	RC4 (mM)		CO_2 evol. (μl)	CO_2 corrected (μl)	Relative activity (%)
substrate; 50 μ moles glucose					
1	0	(no glucose)	32.0		
	0		84.5	52.5	100
	26.8	fresh soln.	76.0	44.0	84
	13.4	〃	80.4	48.8	92
	6.7	〃	84.4	52.4	100
2	0		31.4		
	0		94.5	63.1	100
	13.4	stock soln.	68.6	37.2	59
	6.7	〃	74.7	43.3	69
	3.4	〃	81.7	50.3	80
	1.7	〃	84.0	52.6	83
	0.8	〃	88.0	56.6	90
substrate: 50 μ moles fructose					
3	0	(no fructose)	27.9		
	0		179.4	151·5	100
	26.8	fresh soln.	155.0	127.1	64
	13.4	〃	161.0	133.1	85
	6.7	〃	179.5	151.6	100
4	0		36.7		
	0		144.7	108.0	100
	6.7	stock soln.	98.3	61.6	57
	3.4	〃	104.3	67.6	63
	1.7	〃	116.5	79.8	74
	0.8	〃	118.8	82.1	76
	0.4	〃	147.0	110.3	102

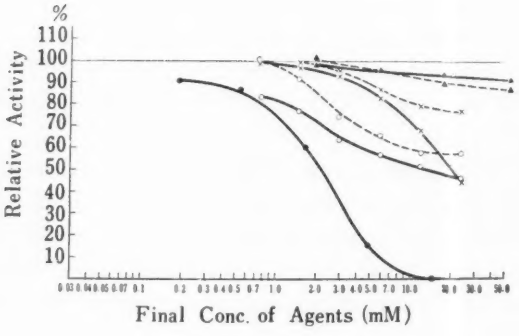


Fig. 5. Relative activity of Oxygen uptake of Ehrlich ascites tumor cells incubated with various concentrations of RC4 or related agents. ×, RC4; ○, o-RC4; ▲, TEPA; ●, NMO. Solid or dotted line indicates the activity of fresh or stock solution of agent, respectively.

Table 2. Effect of RC4 nn respiratory quotient (R.Q.) of Ehrlich ascites tumor cells.

Experiment No.	RC4 (mM)		O_2 uptake (μl)	Relative activity (%)	CO_2 evol. (μl)	Relative activity (%)	R.Q.
1	0		114		101		0.89
	48.3	fresh soln.	31	27	19	19	0.61
	24.2	〃	48	42	31	31	0.64
2	0		107		84		0.79
	48.3	stock soln.	50	47	29	54	0.59
	22.2	〃	73	68	50	59	0.69

solution does not lead to complete inhibition of hexokinase.

Effect on Endogenous Respiration: The mean absolute values for endogenous respiration were Qo_2: 5.7 to 6.4; Qco_2: 5.0 to 5.7; R.Q.: 0.79 to 0.89. The effect of each agent on the consumption of oxygen is shown in Fig. 5. The inhibitory activity of NMO proved to be the highest, 50% at 2×10^{-3} M. and about 10% at 5×10^{-4} M. (Table 2) *o*-RC4 was next to NMO, and the activities of RC4 and TEPA were very low.

Preservation lowered the inhibitory activity of every agent examined, which result was contrary to the case of glycolysis. RC4 showed at a high concentration lowering action of respiratory quotient.

Effect on Human Erythrocyte and Serum ChE: The ChE activity of Ehrlich ascites tumor cells was so low that it was barely measurable even with 2 to 4×10^{-8} cells/bottle. Therefore it was difficult to keep it free from the influence of endogenous respiration, and accurate measurement was impossible. For this reason, human blood was used as ChE source, and the effect of each agent was examined.

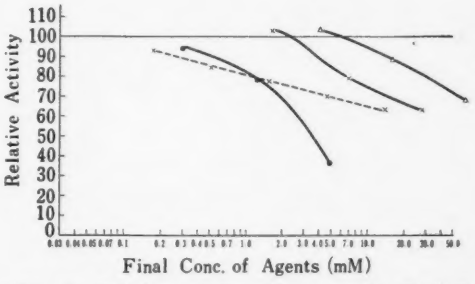


Fig. 6. Relative activity of human red cell cholinesterase incubated with various concentrations of RC4 or related agents.
×——× RC4, fresh solution, ▲——▲ TEPA, fresh solution, ×·····× RC4, stock solution, ●——● NMO, fresh solution

Erythrocyte ChE (specific ChE) and serum ChE (non-specific ChE) were found different in sensitivity to the agents. Upon erythrocyte ChE the most active inhibitor was NMO, RC4 stock solution was next, and RC4 fresh solution as well as TEPA were weak. On the other hand, the most active on serum ChE was RC4 fresh solution, approximately 20% inhibition at 1.5×10^{-3} M and NMO, TEPA and RC4 stock solution displayed approximately equal inhiditory action. In the experiments reported in the

present paper, experiment of serum ChE inhibition was the only case in which the fresh solution of RC4 proved more active than its stock solution and showed its activity even at a considerably low concentration.

Effect on Phosphatase: Under the experimental conditions given in the present report, hydrolysis of PNPP with phosphatase progresses lineally. Either RC4 or TEPA fresh solutions scarcely acted on acid phosphatase, and the activities of their stock solutions were also low. (Table 3) On the other hand NMO, fresh as well as stock solutions,

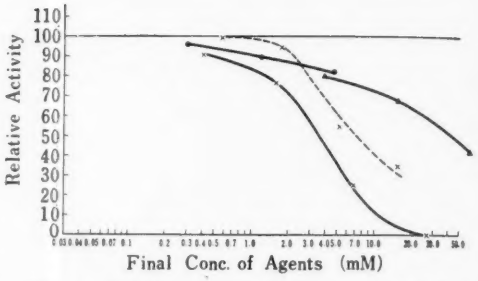


Fig. 7. Relative activity of human serum cholinesterase incubated with various concentrations of RC4 or related agnts.
×——× RC4, fresh solution, ▲——▲ TEPA, fresh solution, ×·····× RC4, stock solution, ●——● NMO, fresh solution.

Table 3. Effect of RC4 or related agents on Ehrlich ascites tumor cell acid-phosphatase.

Experiment	Agent	conc. (mM)	p-Nitrophenol formed (mcq/bottle)	relative activity (%)
1	RC4 fresh solution	0	235	100
		26.8	234	100
		13.4	236	100
2	stock solution	0	240	100
		26.8	214	89
		6.7	220	92
		1.2	235	98
3	NMO fresh solution	0	235	100
		15.8	108	46
		1.8	177	75
		0.2	220	94
4	stock solution	0	155	100
		6.0	77	50
		1.5	131	85
		0.4	150	97
5	TEPA fresh solution	0	233	100
		20.7	226	97
		6.9	232	100
6	stock solution	0	266	100
		20.7	209	79
		6.9	242	91
		2.3	260	97

displayed approximately 20% inhibitory activity at 1.5×10^{-3} M.

While *o*-RC4 displayed a slightly stimulating effect, this compound has an character to intensify the color of *p*-nitrophenol, and considering such influence, the phosphatase-stimulating effect which was observed was within the extent of experimental error.

As regards alkaline-phosphatase the stimulating activity of all the agents but NMO was intensified by preservation. Especially the stimulating effect of RC4 stock solution was outstanding. (Fig. 8) While NMO fresh solution was as stimulative as RC4 stock solustion, its stock solution displayed a powerful inhibitory action.

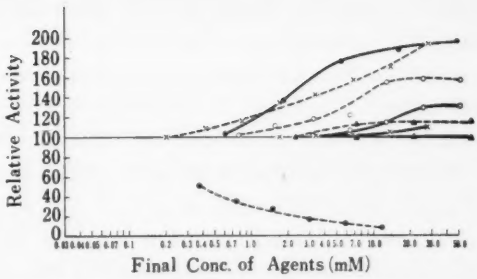


Fig. 8. Relative activity of alkaline-phosphatase of Ehrlich ascites tumor cells incubated with various concentrations of RC4 or related agents. ×, RC4; ○, o-RC4; ▲, TEPA; ●, NMO. Solid or dotted line indicates the activity with fresh or stock solution of agent.

In addition, the effect of m-RC4 on the several enzymes mentioned in the present report was examined, but either its fresh or stock solutions hardly showed any activity. Probably this is due to the exceedingly low solubility of m-RC4.

Discussion

Many students have confirmed that N-mustards inhibit glycolysis, respiration, hexokinase and ChE and stimulate alkaline-phosphatase. As regards ethyleneimines, while the inhibitory activity of TEM[7] or E39[8-10] on glycolysis is considered in connection with their antitumor activities, some other agents in this group are quite weak in glycolysis-inhibiting activity.[8,21] Thus, no consistent results have yet been obtained in regard to ethyleneimines. We have discovered, after examining the characteristics of RC4 and related agents which have ethyleneimino rings, that there are also two different types of such compounds; one group, including o-RC4, immediately displays vigorous activity on anaerobic glycolysis or alkaline-phosphatase at the time of dissolution, while the other group, such as RC4 or TEPA, is very weak in such activity. We have also learned that the activity of the latter group is intensified by preserving their aqueous solutions in a refrigerator and eventually equals that of the former group.

Such a variation in the activity was most typically observed in regard to glycolysis. The increase in glycolysis-inhibiting activity can partly be explained in connection with the increase in anti-hexokinase activity. However, this cannot explain everything, since addition of RC4 stock solution, even in high concentration, does not

inhibit hexokinase completely but does completely inhibit anaerobic glycolysis at a fairy low concentration. Furthermore, despite the anti-glycolysis activity of RC4 solution varies with substrates, its hexokinase-inhibiting activity is not affected by substrates. Therefore, it is presumed that phosphoglucoisomerase may also be inhibited by RC4 stock solution. Probably some other enzymes may partially be inhibited, and the total of all such inhibition may appear as the vigorous inhibition of glycolysis by RC4 stock solution.

The fact that fresh solutions of RC4 and TEPA inhibit serum ChE more severely than erythrocytes ChE, is consistent with Bullock's experiment.[12] He further observed that the inhibitory action of TEM and related agents on specific ChE is intensified under a slightly acid condition. This observation attracts our interest, because we found that the action of RC4 and TEPA on erythrocyte ChE was intensified by preservation in our experiments.

Holzer and his collaborators observed that there were considerable differences among ethyleneimines in the strength of their glycolysis-inhibiting activities, but they did not give sufficient explanation to such differences. In view of the fact that, although there are two groups in RC4 related agents differing in above mentioned activities at the time of dissolution, no such difference is obsereved in their stock solutions, it may be assumed that such divergent activities equally found among ethyleneimines are not essential, but that there is a common active form to reveal the activity and the difference is owing to the variance in velocity of structural change to reach such an active form. This assumption seems to be supported by such facts that, the glycolysis-inhibiting activity of RC4 is intensified along with the opening of ethyleneimno rings and the same phenomenon is observed in TEPA,[22] or that the anti-ChE activity of TEM[12] or the glycolysis-inhibiting activity of E39[8,9] are strengthened on the acid side, on which side opening of the ethyleneimino rings should proceed more easily than under neutral condition.[22]

Then, it may be possible to make clear the significance of the antiglycolsis and other activities of RC4, by comparing the anti-cancer effects of its fresh and stock solutions.

The results of examination of these points are to be presented in forthcoming papers.

Summary

The action of RC4 on the glycolysis, respiration, hexokinase and phosphatase of the Ehrlich ascites tumor cells and on ChE of human blood has been examined in comparison with the actions of o-RC4, TEPA and NMO.

It was observed that, while NMO and o-RC4 displayed strong inhibitorya ctivity against glycolysis and stimulating activity for alkaline phosphatase immediately after

dissolution, RC4 or TEPA were scarcely active on such condition, and, they acquired equal activity to the former two, only after their aqueous solutions were preserved at 2°C.

It might be expected that the clarification of the characteristics of the latter agents will help elucidate those of alkylating agents containing ethyleneimino rings.

We are indebted to Mr. Toru Akiba for his assistance in tumor transplantations.

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[GANN, 51, 201~206; June, 1960]

ELECTROPHORETIC ANALYSIS OF ONCOTREPHIN, THE MITOSIS PROMOTING SUBSTANCE IN MALIGNANT TUMORS

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INTRODUCTION

A substance was isolated from human seminomas and sarcomas, which promotes the mitosis of mouse pinna epidermis *in vitro*. This substance was named oncotrephin (Kuru, Kôsaki, Matuda and Hukui[1]). It was shown to accelerate the growth of L cells in tissue culture (Kôsaki, Matuda, Itô and Kuru[2]). Since the presence of oncotrephin could be demonstrated in ascites hepatomas of rats (Matuda, Hukui, Aoki, Kôsaki and Kuru[3]), an attempt was made to purify it. Some of the results are reported here.

MATERIAL AND METHODS

Rat ascites hepatoma was used. Fractions S_2 and S_2-P_3, which had been shown to promote mitosis of both mouse pinna epidermis and propagation of L cells, were prepared by the procedure described in detail in the previous papers (Matuda, Hukui, Aoki, Kôsaki and Kuru[3]; Itô, Matuda, Kôsaki and Kuru[4]). An aqueous solution of S_2 or S_2-P_3 was further fractionated by continuous paper electrophoresis or by zone electrophoresis. The fractions obtained were divided into several groups and the growth promoting effect of each was compared. For the assay the simplified replicate tissue culture of L cells was used, the details of which were given in the previous papers.[2,4]

RESULTS

1. Effect of fractions separated by continuous paper electrophoresis.

Fraction S_2-P_3 obtained from 300 mg of fraction S_2 was dissolved in 10 ml of distilled water and was exhaustively dialyzed for 24 hours against citrate buffer of ionic strength 0.05 and pH 8.0. The dialyzed solution was then fractionated by continuous paper electrophoresis for 60 hours at 1000v. in the same buffer. The 48 fractions obtained were numbered in order, from the anode to the cathode. Fractions 1-27 were obtained from the anode side and the fractions 28-48 from the cathode side. The protein content of each fraction estimated with Folin-Ciocalteu reagent by the method described by Lowry *et al.*[5] is shown in Fig. 1. Using the protein content

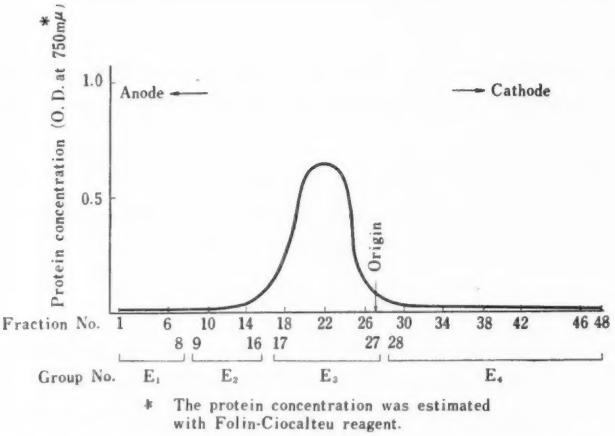

Protein concentration (O.D. at 750mμ)*
1.0
0.5
Anode
Cathode
Origin
Fraction No. 1 6 8 9 10 14 16 17 18 22 26 27 28 30 34 38 42 46 48
Group No. E1 E2 E3 E4
* The protein concentration was estimated with Folin-Ciocalteu reagent.

Fig. 1. Fractionation of fraction S_2-P_3 by continuous paper electrophoresis. Citrate buffer, pH 8.0; μ0.05.

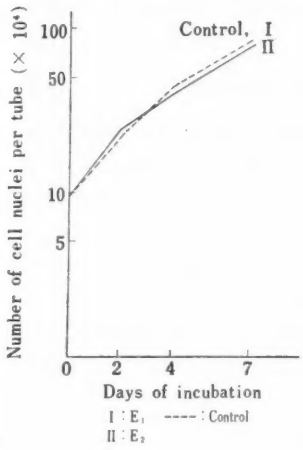


Fig. 2. Effect of groups E_1 and E_2.

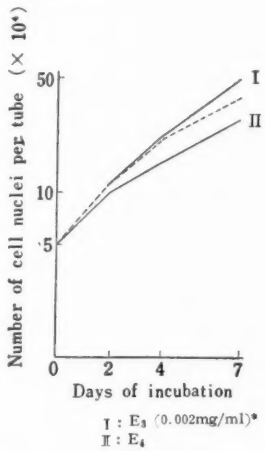


Fig. 3. Effect of groups E_3 and E_4.

as an index, the fractions were divided into four groups. Groups E_1, E_2, E_3 and E_4 correspond to the mixture of fractions 1-8, 9-16, 17-27, 28-48 respectively. Each group was dialyzed against distilled water and lyophilized. Fractions in group E_3 differed from those in the other groups in being orange-yellowish. This group greatly stimulated growth of strain L cells, if added to the culture medium at a concentration of 0.002 mg nitrogen per ml (Fig. 3). The other 3 groups were ineffective

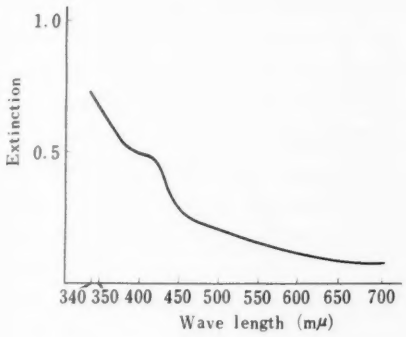


Fig. 4. Absorption spectrum of fraction S_2.

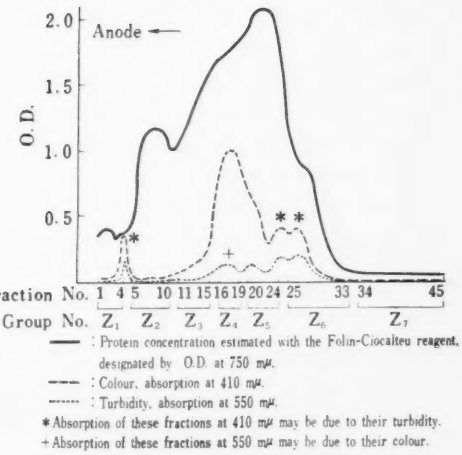

2.0
1.5
1.0
0.5
O.D.
Anode
* *
*
+
Fraction No. 1 4 5 10 11 15 16 19 20 24 25 33 34 45
Group No. Z1 Z2 Z3 Z4 Z5 Z6 Z7
: Protein concentration estimated with the Folin-Ciocalteu reagent, designated by O.D. at 750 mμ.
: Colour, absorption at 410 mμ.
: Turbidity, absorption at 550 mμ.
* Absorption of these fractions at 410 mμ may be due to their turbidity.
+ Absorption of these fractions at 550 mμ may be due to their colour.

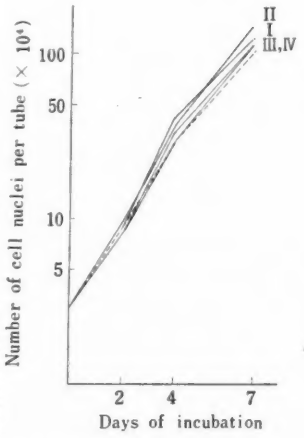
Fig. 5. Fractionation of fraction S_2 by zone electrophoresis in a vertical starch column. Veronal buffer, pH 8.6; μ 0.05.

at a similar concentration (Fig. 2 and 3).

2. Effect of fractions separated by zone electrophoresis.

As above-mentioned, the effective substance was shown to move to the anode at pH 8.6 on continuous paper electrophoresis. However, as the yield of each fraction by this method was very low, zone electrophoresis was tried. Fractionation by zone electrophoresis was repeated 3 times with consistent results. One of these three fractionations is described here.

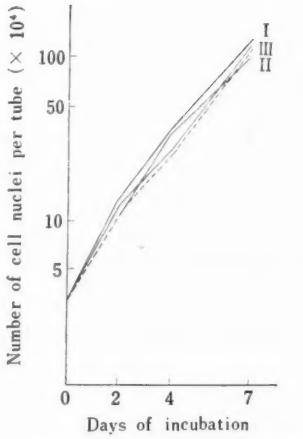
540 mg of fraction S_2 was dissolved in 7 ml of veronal buffer (pH 8.6, μ 0.05). The solution was tinged orange-yellowish and the spectrum showed maximal absorption at 406 mμ (Fig. 4). This corresponds fairly closely to the Soret band, suggesting that it resulted from a porphyrin compound. Zone electrophoresis was undertaken using a vertical starch column in the same buffer for 66 hours at 3°C with a constant current (15 mA). Immediately after electrophoresis, material was eluted with the same buffer. The eluate was collected in 45 fractions and these were divided into 7 groups as shown in the diagram in Fig. 5. In this diagram the protein content of each fraction is shown as estimated with the Folin-Ciocalteu reagent. Turbidity was measured by the extinction at 550 mμ, and the extent of absorption at 410 mμ corresponded to the intensity of orang-yellowish colour. Groups Z_1, Z_2, Z_3, Z_4, Z_5, Z_6 and Z_7 correspond to fractions 1-4, 5-10, 11-15, 16-19, 20-24, 25-33 and 34-45 respectively. The 7 groups of pooled eluate were thoroughly dialyzed against distilled water at low temperature for *ca.* 80 hours to remove the veronal. After dialysis, subgroup Z_5', was separated from group Z_5 by centrifugation, because of its insolubility



I : 0.01mg/ml* IV : 0.00008mg/ml*
II : 0.002mg/ml* ---: Control
III : 0.0004mg/ml*

*Final concentration of test material added to the culture medium, in mg N.

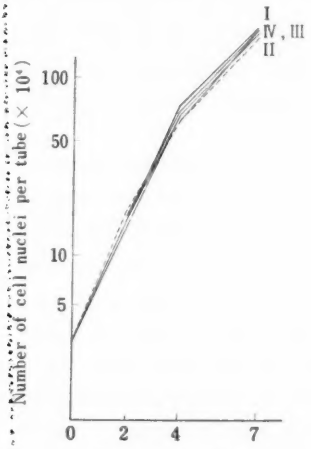
Fig. 6. Effect of group Z_5.



I : 0.002mg/ml* III : 0.00008mg/ml*
II : 0.0004mg/ml* ---: Control

*Final concentration of test material added to the culture medium, in mg N.

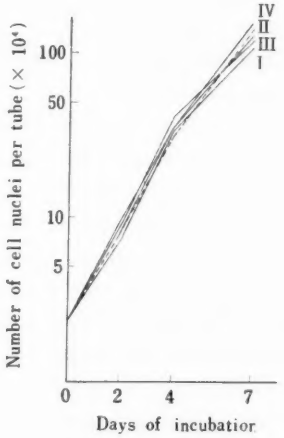
Fig. 7. Effect of group Z_4.



: 0.01mg/ml* III : 0.0004mg/ml*
: 0.002mg/ml* IV : 0.00008mg/ml*
---: Control

*Final concentration of tset material in the culture medium, as mg N.

Fig. 8. Effect of group Z_5'.



I : 0.01mg/ml* III : 0.0004mg/ml*
II : 0.002mg/ml* IV : 0.00008mg/ml*
---: Control

*Final concentration of test material in the culture medium, as mg N.

Fig. 9. Effect of group Z_3.

in solutions of low ionic strength at pH 5.8. After neutralization, the sediment was dissolved in distilled water at pH 7.0. All the groups were lyophilized. Group Z_5, which contained those fractions having the peak of the protein in the electrophoregram and characterized by its faint orange-yellowish colour, markedly stimulated the propagation of L cells, if added to the culture medium at a concentration of 0.002mg nitrogen per ml (Fig. 6). In group Z_4, however, in which the orange-yellowish colour was most conspicuous, a growth promoting effect was scarcely recognizable (Fig. 7). No other group, inclusive of subgroup Z'_5, was effective at a similar concentration (Fig. 8, 9).

3. Test of the electrophoretic mobility of each fraction by paper electrophoresis.

As zone electrophoresis fractions 20–24 (group Z_5) were shown to be effective and to involve the peak of the protein in the electrophoretic pattern, the mobility of the protein in these fractions was investigated by paper electrophoresis. Protein which moved with a velocity a little slower than that of human β-globulin at pH 8.6, could be demonstrated in them (Fig. 10). Under the same conditions group Z_2 migrated a little faster than human serum albumin and group Z_6 a little faster than human γ-globulin.

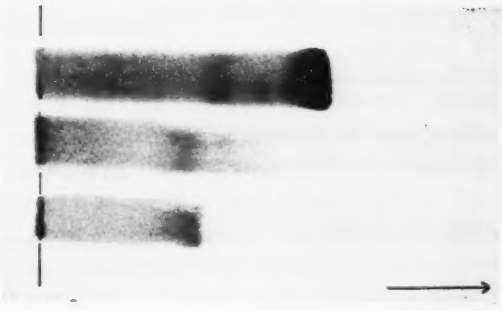


Fig. 10. Paper electrophoresis of fraction S_2, group Z_5 and human serum. Upper: Human serum; Middle: Fraction S_2; Lower: Group Z_5. Veronal buffer, pH 8.6; μ 0.05.

Conclusion

Fraction S_2 from rat ascites hepatoma, which was shown to promote the growth of strain L cells in tissue culture, was further fractionated by continuous paper electrophoresis and zone electrophoresis. The fractions obtained were divided into several groups according to their protein content and the effect of each group upon propagation of L cells was investigated. Results were as follows:

1) The effective substance is localized exclusively in certain fractions. These fractions are distinguishable from others by their high protein content. The bulk of the protein in this group moves a little slower than human β-globulin on paper electrophoresis at pH 8.6.

2) These fractions are also characterized by their faint orange-yellowish colour. This colour is related to a porphyrin compound though it is not known whether

heme is essential for the growth-promoting effect.

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[GANN, 51, 207~212; June, 1960]

ISOLATION OF AN ONCOTREPHIN-LIKE SUBSTANCE FROM REGENERATING RAT LIVER

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INTRODUCTION

In the previous communications the authors (Kuru, Kôsaki, Matuda and Hukui (1959)[13]) have shown that a substance can be isolated from certain human malignant tumors, which promotes the mitosis of mouse pinna epidermis *in vitro*. This substance, which was named oncotrephin, was further shown to promote the growth of L cells in tissue culture (Kôsaki, Matuda, Itô and Kuru (1959)[12]). Since oncotrephin was shown to be present in rat ascites hepatoma (Matuda, Hukui, Aoki, Kôsaki, and Kuru (1959)[16]), its purification was attempted as reported in the preceding paper (Kuru, Kôsaki, Matuda and Itô (1960)[14]).

The present paper reports results of studies on the isolation of an oncotrephin-like substance from regenerating liver.

MATERIALS AND METHODS

Two-thirds of the liver tissue of hybrid rats were removed under ether anesthesia by the standard procedure (Higgins and Anderson (1931)[10]). After 72 hours the animals were sacrificed. The livers were perfused with saline to remove as much blood as possible and taken out. They were then homogenized with 3 parts of 0.95% saline in a Waring blender, kept overnight in a refrigerator and centrifuged at 10000 r.p.m. for 15 minutes at 0°-5°C. The supernatant, crude extract, was fractionated in the same way as in the isolation of oncotrephin from rat ascites hepatoma, and 3 fractions were obtained, S_1, S_2, S_3 which were the precipitate with 30 volume per cent ethanol, the precipitate with 30-70 volume per cent ethanol and the supernatant, respectively.

Analogous fractions were obtained from normal rat liver as controls.

These fractions were added to the culture media at various concentrations and their effect studied. For the estimation of their growth-promoting effect, a simplified replicate tissue culture method was used employing L cells. The details of this method have been given in the previous papers (Kôsaki, Matuda, Itô and Kuru,[12]

Itò, Matuda, Kôsaki and Kuru[11]).

Results

1) Effect of fractions obtained from normal liver.

As reported in the previous paper[11], at high concentrations (0.05–0.01 mg nitrogen per ml), fraction S_2 from normal liver inhibits the growth of L cells. At lower concentrations no growth-promoting effect can be observed (Fig. 1). The growth-promoting effect of fractions S_1 and S_3 from normal liver at a concentration of 0.01–0.002 mg nitrogen per ml, is almost negligible (Fig. 2).

2) Effect of fractions obtained from regenerating liver.

Fraction S_2 from regenerating liver greatly promotes the growth of L cells at a final concentration of 0.002–0.00008 mg nitrogen per ml. At a higher concentration (0.05 mg nitrogen per ml) it exerts an inhibitory influence upon propagation of L cells (Figs. 3, 6).

Fractions S_1 and S_3 have no effect upon the growth of L cells at a concentration of 0.01–0.002 mg nitrogen per ml (Figs. 4, 5).

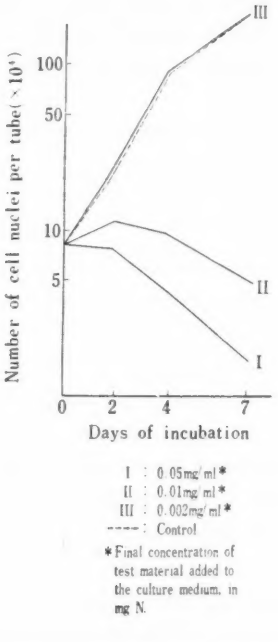


Fig. 1. Effect of fraction S_2 from normal rat liver on proliferation of L cells.

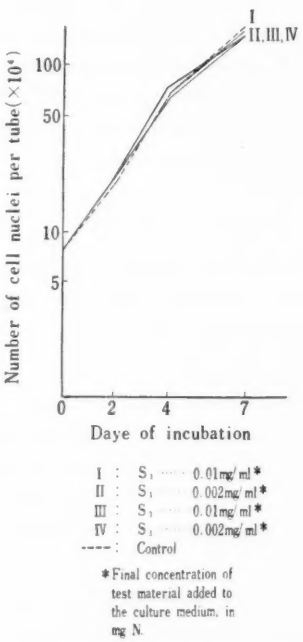
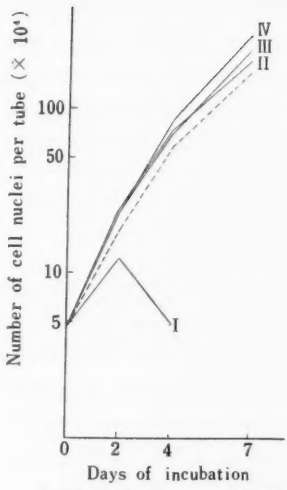


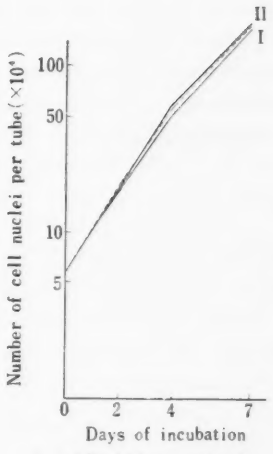
Fig. 2. Effect of fractions S_1 and S_3 from normal rat liver on proliferation of L cells.



I : 0.05 mg/ml*　　IV : 0.0004 mg/ml*
II : 0.01 mg/ml*　　---- : Control
III : 0.002mg/ml*

*Final concentration of test material added to the culture medium, in mg N.

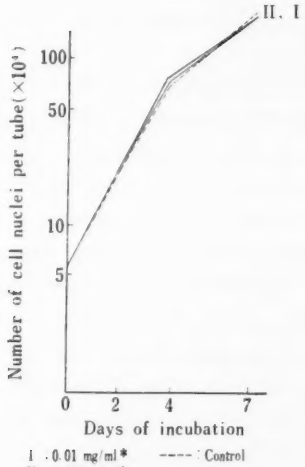
Fig 3. Effect of fraction S_2 from regenerating rat liver on proliferation of L cells.



I : 0.01mg/ml*　　---- : Control
II : 0.002mg/ml*

*Final concentration of test material added to the culture medium, in mg N.

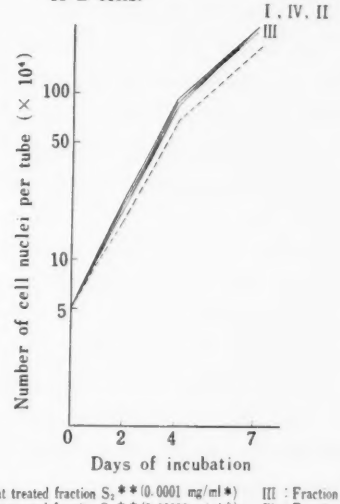
Fig. 4. Effect of fraction S_1 from regenerating rat liver on proliferation of L cells.



I : 0.01 mg/ml*　　---- : Control
II : 0.002mg/ml*

*Final concentration of test material added to the culture medium, in mg N.

Fig. 5. Effect of fraction S_3 from regenerating rat liver on proliferation of L cells.



I : Heat treated fraction S_2** (0.0001 mg/ml*)　　III : Fraction S_2 (0.0004 mg/ml*)
II : Heat treated fraction S_2** (0.00002mg/ml*)　　IV : Fraction S_2 (0.00008mg/ml*)
----: Control

* Final concentration of test material added to the culture medium, in mg N.

** Heated 30 minutes in a boiling water bath, centrifuged at 10000 r.p.m. for 10 minutes, then passed through a membrane filter.

Fig. 6. Effect of heat treated fraction S_2 from regenerating rat liver on proliferation of L cells.

3) Influence of heat upon the effect of fraction S_2 from regenerating liver.

An aqueous solution of fraction S_2 from regenerating liver was heated in a boiling water bath for 30 minutes and then centrifuged at 10000 r.p.m. for 10 minutes. The supernatant was added to the culture medium at a final concentration of 0.0001–0.00002 mg nitrogen per ml. The same stimulatory effect was seen as with the unheated material (Fig. 6).

Discussion

In response to biological demand, regenerative tissues can grow rapidly, even under unfavorable circumstances; this character strongly resembles that of malignant tumors. Consequently, regenerating tissues and malignant tumors have many common histological features. The lack of polarity of the constituent cells, the hyperchromatism of the nuclei, and increased appearance of mitotic figures are common features encountered in microscopical preparations. The main characteristic which distinguishes regenerating tissues from malignant tumors is the presence of reversibility. In regeneration, as soon as restoration is accomplished, the accelerated growth stops, while in malignant tumors the uncontrolled growth proceeds indefinitely.

In both processes, the existence of a certain growth promoting substance (or substances) has been postulated repeatedly. A search for such substances was initiated by Carrel (1912–1926)[3,4,5,6], who described "trephone" as an active substance. This search was unfruitful, until recently when regenerating liver became the subject for detailed investigations.

Christensen and Jacobsen (1950)[7], Bucher, Scott and Aub (1951)[2] and also Wenneker and Sussman (1951)[20] have demonstrated that the partial hepatectomy of one member of a parabiotic pair stimulates mitotic activity of the liver of the other intact parabiont. Glinos and Gey (1952)[9] have shown that the serum from partially hepatectomized rats enhances growth of both a primary liver explant and a normal fibroblast strain in tissue culture. Friedrich-Freksa and Zaki (1954)[8] have found that serum from a partially hepatectomized rat, if injected intraperitoneally into another rat, markedly enhances mitotic activity of the liver. They state that the effective substance is nondialysable and organ-specific. Another organ-specific mitosis-promoting agent was described by Ogawa and Nowinski (1958)[17]. They demonstrated that the serum of a unilaterally nephrectomized rat, when added to the culture medium, accelerates the growth of epithelial-like cells of rat kidney in tissue culture. They consider that this substance is not species-specific, and is inactivated by heating for 20 minutes at 100°C. Blomqvist (1957)[1] has shown that intraperitoneal injection of a homogenate of regenerating liver actually stimulates mitotic activity of liver. He further showed development of malignant abdominal tumors following long-term

injection of a homogenate of regenerating liver. One of these tumors proved to be an anaplastic liver tumor.

The growth-promoting substance in liver itself was also studied by certain investigators. Teir and Ravanti (1953)[19] stated that intraperitoneal injection of suspensions of liver tissue clearly stimulates the mitotic ratio in the liver. With suspensions from younger animals the effect is greater than with those from old animals. They consider this mitosis-promoting agent to be thermolabile and organ-specific.

Previous to these investigations, Marshak and Walker (1945)[15] succeeded in isolating a mitosis-stimulating substance from liver. This substance was found in the chromatin fraction. In their experiments hepatectomized rats were used for demonstration of increased mitosis and test materials were administered intravenously.

The experiments of Paschkis, Cantarow, Stasney and Hobbs (1955)[18] are of interest. They studied a possible competition between the tumor and regenerating liver. They found that the growth of Walker tumor 256 and of a transplantable hepatoma (but not Jensen sarcoma and Murphy lymphosarcoma) was significantly greater in partially hepatectomized rats than in intact animals. They concluded that the regenerating liver may release a hormonal agent causing increased growth of some tumors. It seems likely that liver regeneration too, was enhanced by growing tumors. However they hesitated to assume a causal relation in this case.

The present authors[11,12,13,16] have succeeded in demonstrating a substance, oncotrephin, in rapidly growing malignant tumors, which promotes the mitosis of mouse pinna *in vitro* and simultaneously promotes the propagation of L cells in tissue culture. In the present study the existence of an analogous substance in regenerating liver was demonstrated. Purification of this substance, with special reference to its identification with the oncotrephin, is now in progress.

Conclusion

The fraction from a crude extracts of rat regenerating liver, which is precipitated by 30–70 volume per cent ethanol, contains an oncotrephin-like substance. That is to say, it remarkably stimulates growth of L cells in tissue culture, if added at an optimal concentration. Like oncotrephin, the effective substance is thermostable.

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